Therapeutic Targets for Cholestatic Liver Injury

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Abstract

Introduction—Cholestasis is a reduction in bile flow that occurs during numerous pathologies. Blockage of the biliary tracts results in hepatic accumulation of bile acids or their conjugate bile salts. The molecular mechanisms behind liver injury associated with cholestasis are extensively studied, but not well understood. Multiple models of obstructive cholestasis result in a significant inflammatory infiltrate at the sites of necrosis that characterize the injury.

Areas Covered—This review will focus on direct bile acid toxicity during cholestasis, bile acid signaling processes and on the development and continuation of inflammation during cholestasis, with a focus on novel proposed molecular mediators of neutrophil recruitment. While significant progress has been made on these molecular mechanisms, a continued focus on how cholestasis and the innate immune system interact is necessary to discover targetable therapeutics that might protect the liver while leaving global immunity intact.

Expert Opinion—While bile acid toxicity likely occurs in humans and other mammals when toxic bile acids accumulate, persistent inflammation is likely responsible for continued liver injury during obstructive cholestasis. Targeting molecular mediators of inflammation may help prevent liver injury during acute cholestasis both in murine models and human patients.

Keywords

Obstructive cholestasis; bile acid toxicity; inflammation; innate immunity; neutrophils

1. Introduction

Cholestasis results from blockage of the biliary tracts at any point or due to inhibition of the bile salt export pump (BSEP) at the canalicular surface of the hepatocyte. This blockade results in accumulation of bile acids (BA) or their conjugate salts in hepatocytes and in serum. While some bile acids or bile salts such as glycochenodeoxycholate (GCDC) are highly toxic [1], many of the bile acids that accumulate *in vivo* in rodent models during cholestasis are largely non-toxic, even up to biliary (millimolar) concentrations [2]. Exposure of isolated hepatocytes to serum and biliary concentrations of bile acids that are

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observed during cholestasis *in vivo* is largely non-toxic, unless the bile acid composition is toxified through the addition of hydrophobic bile acids like GCDC or lithocholic acid (LCA) to the system [3]. As such, new hypotheses have been put forth on the mechanisms behind what causes hepatocyte cytotoxicity during cholestasis [4]. While there are some discrepancies between models dependent on the cause of cholestasis, neutrophils have been implicated as critical to the pathogenesis of multiple models of cholestatic liver injury [4,5,6]. This review will focus largely on obstructive cholestasis and the bile duct ligation (BDL) model of injury and the signaling mechanisms and inflammation associated with this model. While there are other relevant models, a plurality of the papers on cholestatic liver injury take place using the BDL model as it recapitulates many facets of human obstructive cholestasis. It should be noted that while the BDL model is an excellent model for the basic physiology and pathophysiology of the injury, pharmaceutical intervention in the model can be prone to problems, thus, this review will focus mainly on basic science and interventions that use knockout mouse models that do not suffer from this problem [8]. How bile acid accumulation and the innate immune system interact, the makeup of the chemotactic signals that recruit neutrophils to the liver, and the mechanisms behind how neutrophils kill hepatocytes during cholestasis remain unanswered questions. This review will address these questions as well as offer current hypotheses on the role and causes of sterile inflammation during cholestatic liver injury, and review potential therapeutic targets related to bile acid signaling as it is currently understood.

2. Bile Acid Signaling During Cholestasis and its Relation to Innate Immunity

2.1 Bile acid accumulation during cholestasis

A considerable number of papers have focused on bile acid accumulation as the primary mechanism of cellular toxicity during cholestasis in rodents [1]. This hypothesis is based upon the administration of GCDC in rodent hepatocytes [9, 10] or transfected hepatoma lines [11, 12]. Administration of 50–100 μM GCDC to these lines results in well-defined apoptotic cell death that is inhibitable by pan-caspase inhibition [13]. The use of these concentrations of GCDC was originally predicated upon GCDC accumulation during cholestasis in humans, and its recognized toxicity [9,10]. However, more recent reports showed that *GCDC accumulation during cholestasis is largely limited to man*, and rodents accumulate much higher concentrations of muricholic acid (MCA), a secondary derivative of chenodeoxycholic acid (CDCA) [2, 14]. Other commonly used bile acids to induce toxicity include unconjugated CDCA or DCA; however, 99% or more of the circulating and liver levels of these bile acids found during cholestasis are typically conjugated to either taurine or glycine, thus, the use of micromolar concentrations of unconjugated bile acids may be unwarranted [2]. Also of note, the idea of bile acid-induced apoptosis in rat hepatocytes may be contingent upon unrealistic exposure of hepatocytes to normoxic air, as simulated exposure to physiological hypoxia experienced in the liver resulted in protection against GCDC induced apoptosis [15]. This may be due to the excess oxygen, which is responsible for the creation of reactive oxygen species under normoxic conditions present during *in vitro* cell culture [16]. Moreover, a majority of bile acids are conjugated to taurine during cholestasis in rodent species, which produces a less toxic bile acid mixture [2]. This is in

contrast to man where glycine conjugates, which are far more toxic, occur in approximately equal frequency to taurine conjugates [2, 14, 17]. Taurocholic acid (TCA), MCA and its conjugated derivatives α- and β-tauromuricholic acid compose a majority of the bile acid pool during cholestasis in mice, and display essentially no toxicity *in vitro* towards hepatocytes, even at millimolar concentrations [2, 18]. In contrast, GCDC levels never accumulate to micromolar levels in serum of rodent models after BDL, nor do they accumulate in the liver to levels on par with toxicity [2]. While rat hepatocytes will undergo apoptosis at concentrations as low as 50μM, human hepatocytes do not undergo apoptosis in response to GCDC, and will only undergo necrosis at concentrations of 500μM and above [19, 20]. These data strongly argue against the idea that exposure of rodent hepatocytes to GCDC mimics the human pathological condition. Instead, in humans biliary concentrations of bile acids associated with hepatic infarction directly induce cell death, which leads to further inflammatory liver injury [20]. Critical to understanding of the injury is how the accumulation of bile acids in hepatocytes leads to a sustained chemotactic signal and predisposes these hepatocytes towards a neutrophilic attack.

2.2 Bile acid signaling in hepatocytes and innate immune cells of the liver

Cholestatic concentrations of bile acids induce a number of genetic changes in hepatocytes [21]. In the BDL model, hepatocytes acutely upregulate export transporters such as the multidrug resistance (Mdr) transporter family and BSEP [22] and downregulate uptake transporters, especially the sodium taurocholate cotransporting polypeptide (Ntcp), in order to reduce hepatic accumulation of bile salts [23]; although, post-transcriptional regulation of Bsep results in a reduction in total activity [24]. This is likely due to an inherent need to reduce bile acid transport into bile because of the lack of bile flow [24]. In Mdr3 (Abcb4)-deficient mice similar results are observed, wherein the mice have increased Bsep mRNA levels after birth; however, total protein expression levels of Bsep are significantly lower [25]. Generally, these compensatory changes are thought to serve as a way to protect hepatocytes from hepatic accumulation of bile acids and shunt excess bile acid levels to the serum where they can potentially be taken up and excreted by the kidneys [24, 26]. A number of these actions take place through autoregulation of bile acid enterohepatic circulation, synthesis, and metabolism via the endogenous bile acid nuclear receptor Farnesoid X Receptor (FXR). Bile acids serve as ligands that activate FXR, which responds by upregulating and downregulating the aforementioned genes, as well as CYP7A1, the rate-limiting enzyme in the classical bile acid synthesis pathway. FXR has been studied extensively and is reviewed in depth elsewhere [27]. FXR is a critical response element to cholestatic concentrations of bile acids, as FXR$^{-/-}$ mice cannot upregulate Bsep to enhance choleresis and undergo increased injury in response to cholestasis [28]. Currently a specific FXR agonist, obeticholic acid, is undergoing clinical trials as a potential therapeutic in the treatment of primary biliary cirrhosis (PBC), a form of autoimmune cholestatic hepatitis [29]. Promisingly, in patients with an inadequate response to UDCA, the current mainline treatment, obeticholic acid treatment resulted in decreases in alkaline phosphatase, γ-glutamyl transpeptidase and alanine aminotransferase, suggesting obeticholic acid can protect hepatocytes, and biliary epithelial cells against PBC-associated liver damage [29]. It should be noted that due to the complete biliary obstruction induced in the BDL model, other choleretic drugs such as ursodeoxycholate (UDCA), the mainline cholestatic

therapeutic, can actually worsen injury as they contribute to increased biliary pressure and infarction of the biliary tracts [30, 31]. Doses given in man probably fail to exacerbate the injury due to the smaller quantities, and the protective nature of UDCA towards the mitochondria [19]; thus, these differences should serve as a cautionary note that the BDL model may not always be appropriate to test therapeutics for cholestatic liver injury.

Bile acids can also activate extracellular receptors. Bile acids have been shown to activate both receptor tyrosine kinases and G-protein coupled receptors (GPCRs) on hepatocytes [32, 33] and also activate downstream MAPK proteins via activation of the epidermal growth factor receptor [34]. While many of these signaling cascades seem to be dependent on ROS generation and injury in rat hepatocytes [33], it is not well known if these mechanisms are recapitulated in other mammals. Bile acids also act as ligands for sphingosine 1 phosphate receptor 2 (S1PR2) in isolated rat hepatocytes, which activates MAPK signaling in hepatocytes [35]. Although S1PR2 inhibition was not protective against bile acid-induced cytotoxicity, inhibition of sphingosine kinase or administration of exogenous sphingosine 1 phosphate (S1P), were both protective against cell death *in vitro* [36]. Interestingly, while S1PR3 antagonism has not been examined *in vitro*, inhibition of S1PR3 has been shown to inhibit S1P mediated bone marrow derived cell homing and reduce fibrosis and injury during cholestasis *in vivo* in the BDL model [37]. Although the detailed mechanisms of how this occurs have yet to be determined, S1PR activity may be a potential therapeutic target to reduce neutrophil accumulation during cholestasis without compromising global immunity.

The recently discovered G-protein coupled bile acid receptor (GPBAR or hereafter referred to as TGR5) is a novel receptor for bile acids expressed on multiple different cell types [38]. TGR5 is expressed on monocytes, sinusoidal endothelial cells, Kupffer cells, cholangiocytes, and to a lesser degree, hepatocytes in the liver [38–40]. Due to the diverse expression pattern, TGR5 activation by bile acids has pleiotropic effects, including varied effects in multiple models of liver injury such as cholestasis [41], LPS-induced inflammation [42], and partial hepatectomy [43], in addition to native physiological functions such as gallbladder filling [44]. TGR5 activation is thought to be generally protective against liver injury in multiple ways. TGR5 activation on sinusoidal endothelial cells induces endothelial nitric oxide synthase and nitric oxide (NO) production via cyclic adenosine monophosphate (cAMP) [45]. NO is a critical mediator of vasodilation which may serve to prevent portal hypertension, one of the most serious complications of cirrhosis caused by long term cholestasis. TGR5 activation in macrophages and Kupffer cells antagonizes NF-kB mediated increases in pathogenic cytokines during cholestasis [42]. This leads to protection against injury, as TGR5 KO mice have exacerbated inflammation after BDL or partial hepatectomy, particularly, an increase in recruited neutrophils [43]. Kupffer cell depletion through clodronated liposomes attenuated the TGR5 KO phenotype, suggesting a portion of this phenotype is due to TGR5 mediated ablation of pro-inflammatory Kupffer cell activity [43]. TGR5 is also known to enhance biliary excretion and bile flow by enhancing bicarbonate output, and thus helps control bile acid overload in models of cholestasis and liver regeneration [41, 43]. In addition to obeticholic acid, a TGR5/FXR dual agonist (INT-767) is in development that might not only enhance choleresis, but also limit inflammation that occurs concurrently during liver disease. As macrophages are constantly exposed to serum bile acids levels, TGR5 activation by excess serum bile acids may be beneficial in the

greater context of injury. Treatments that push bile acids outside of the enterohepatic circulation and into systemic circulation not only bypass cholestatic, damaged areas, but may also serve to enhance systemic bile acid elimination via the kidneys and activate protective measures through TGR5 on macrophages. It should be noted though, that chronic cholestasis and enhanced renal bile acid excretion has been associated with cholemic nephropathy [26]. Thus, more studies would be required to determine if renal excretion is a viable route to enhance elimination of excess bile acids levels without renal toxicity.

Recent reports suggest that bile acids themselves directly cause innate immune activation and recruitment in addition to their other processes [18, 46]. Mouse hepatocytes exposed to pathophysiological levels of multiple conjugated bile acids respond by upregulating C-X-C motif chemokines such as CXCL-1 (referred to as mKC) and CXCL-2 (referred to as MIP-2) in an FXR-*independent* manner [18]. This response is potentially dependent on bile acid uptake as this response is lost if hepatocytes are allowed to adhere for 24 hours before the initial exposure, (Woolbright and Jaeschke, unpublished observation) a period during which cultured primary rodent hepatocytes naturally dedifferentiate, and downregulate Ntcp and other uptake transporters [47, 48]; although, dedifferentiation of hepatocytes could have other additional unknown effects independent of the decrease in Ntcp that might account for this loss of response. In addition to cytokines, bile acids can directly upregulate the transcription factor early growth response protein 1 (Egr-1) [49]. This action is mediated by MAP kinases, and thus may be linked to the aforementioned activation of cell surface receptors [49]. Egr-1 is also upregulated by BDL, and is essential for inflammation after BDL, as Egr-1$^{-/-}$ mice are largely protected from BDL-induced liver injury [50]. Egr-1$^{-/-}$ mice fail to upregulate cytokines in response to bile acid exposure, linking the *in vitro* upregulation of cytokines and pro-inflammatory proteins like intercellular adhesion molecule-1 (ICAM-1) to obstructive cholestasis and inflammation [18]. IL-17A may act on the same axis as it synergistically enhances Egr-1 signaling, increasing cytokine production [46]. IL-17 has been shown to enhance both immune activity and progression of fibrosis during obstructive cholestasis [51], potentially linking bile acid-induced inflammation directly to downstream fibrosis. Thus, Egr-1 may play a central role in the inflammatory process during cholestasis and is a potential therapeutic target for inflammation during cholestatic liver injury. However, it should be noted that studies in human hepatocytes have indicated a considerably blunted inflammatory response compared to mouse hepatocytes [20]. These findings are illustrated in Figure 1.

3. Inflammation as a Mechanism of Injury during Cholestasis

3.1 The innate immune system

The primary role of the immune system is host defense against microbial pathogens. Multiple components of the innate immune system rapidly respond to microbial infection to identify, phagocytose, and kill microbial components. The liver is a critical organ in the innate immune defense response, as Kupffer cells in the liver filter a substantial amount of bacteria, LPS, and viral components from the blood stream. Sterile inflammation parallels host defense, wherein the inflammatory system activates against normal cellular components that are perceived as indicators of cellular damage termed *damage associated molecular*

patterns (DAMPs), instead of activating against microbial pathogens. This duality is critical to understand. Global immunosuppression to prevent injury during liver disease is not a realistic therapeutic option, as the risk of infection can become greater than the therapeutic benefit. Thus, there is an impetus to identify therapeutic targets that prevent inflammation during cholestatic liver injury without compromising the global immune response. This section will focus on new mediators of neutrophil extravasation with the potential to inhibit inflammation after cholestasis while minimizing global impact, and also discuss current mechanisms of innate immune cell recruitment and injury.

3.2 The role of neutrophils during cholestatic liver injury

Neutrophils are a critical component of the innate immune system and the most populous granulocytes in mice and man. Initially, neutrophils were shown to correlate with the areas of biliary infarction and focal necrosis that characterize obstructive cholestasis [52]. Subsequently, BDL was shown to cause an increase in chemoattractants in the liver [53]. Mice deficient in ICAM-1 or CD18, which are important for neutrophil extravasation and cytotoxicity [54], are both protected against BDL-induced liver injury [5, 55]. In addition, depletion of P-selectin glycoprotein ligand 1 attenuated neutrophil-mediated injury after BDL [56]. Anti-inflammatory glucocorticoids are protective against BDL in rats [57]. Neutrophils have also been found to mediate injury in some models of intrahepatic cholestasis [6, 58]. In neutrophil-depleted mice fed alpha-naphthylisothiocyanate (ANIT) there was a substantial decrease in injury that could be directly attributed towards protection of hepatocytes, and not protection of cholangiocytes, in line with data in the BDL mouse [6]. Neutrophils express myeloperoxidase, which is responsible for the generation of hypochlorous acid, a potent oxidant. The areas of biliary infarction that characterize the focal necrosis immunostain positive for chlorotyrosine adducts, indicative of release of the highly toxic hypochlorous acid in the region [5, 55, 59]. While these data support a hypothesis where neutrophils are the predominant source of injury, the idea that neutrophils caused cholestatic liver injury was controversial. Previously, the injury was thought to be largely apoptotic through a mitochondrial-lysosomal axis, as both cathepsin $B^{-/-}$ mice and fas receptor-deficient *lpr* mice were protected against BDL [60, 61]. Of note, a reduction in neutrophil recruitment was observed in the cathepsin $B^{-/-}$ mouse, though the mechanism was never discerned [61]. Furthermore, the *lpr* mouse was found to have a blunted inflammatory response to BDL, and pan-caspase inhibition was shown to have no effect on the injury [62]. These findings suggested necrosis is the dominant mode of cell death. Subsequent studies investigating the modality of cell death further confirmed focal necrosis, eliminating the idea that apoptosis could be the primary mechanism of injury *in vivo* [63–65]. Together, these data strongly indicate that neutrophils are the primary source of toxicity after BDL. Given that bile acid concentrations in murine bile are insufficient to result in toxicity [2, 18] the current hypothesis is that neutrophils are recruited to the liver, where they target, and kill, injured hepatocytes. This mechanism involves bile acids as pro-inflammatory but not directly cytotoxic mediators [4]. These findings are illustrated in Figure 2.

While neutrophils are the predominant innate immune cell during cholestasis, there are significant interactions with other components of the innate immune system. While Kupffer

cell inactivation with gadolinium chloride was initially thought to be protective against BDL-induced injury [66], this was later attributed to be an effect of gadolinium chloride itself and likely unrelated to Kupffer cell activation, as depletion of Kupffer cells with the relatively more specific clodronate liposomes resulted in an enhancement of BDL injury [67]. Kupffer cells activate NF-κB which results in IL-6 production during early cholestasis that can protect against injury [67]. Adaptive immune cells contribute to the inflammatory signal as well, as both natural killer cells and invariant natural killer T cells help to suppress cholestatic liver injury through this same function, as they activate protective effects in Kupffer cells [68, 69]. A majority of the effects of these other immune components are likely achieved through modulation of neutrophil recruitment though, as neutrophils carry out the actual cell killing processes.

3.3 Novel Mediators of Neutrophil Recruitment in Cholestasis

As global inflammatory inhibition is not a viable therapeutic avenue, identifying novel pathways of neutrophil recruitment during cholestasis are necessary in order to find specific pharmaceutical targets. Numerous advances have occurred recently in the field of sterile inflammation. Amongst these is the identification of receptors of DAMPs that result in activation of immune cells and stimulation of sterile inflammatory response [70]. Numerous DAMPs have been identified thus far, as well as nine receptors (toll-like receptors, TLRs) for these ligands [70]. Many DAMPs are released passively from necrotic cells, such as mitochondrial DNA, nuclear DNA fragments, and ATP [71–74]. DAMP release has been proposed as both a clinical and a mechanistic biomarker that may give additional insight into clinical injury, without the need for directly sampling the injured tissue [72, 73, 75–76]. Since acute liver injury compromises the coagulation system and poses a serious bleeding risk, the use of serum biomarkers provides an interesting and suitable alternative to tissue studies in man. One such serum biomarker is HMGB1, which binds and activates TLR4 [71]. HMGB1 is a DNA associated histone deacetylase that translocates to the cytosol during injury [77]. Necrosis during cholestasis results in release of HMGB1 into serum as early as 6 hours after injury [65]. In addition to native HMGB1, an acetylated form is actively secreted from macrophages that may serve as a pro-inflammatory signal [78]. The ratio of acetylated to hypoacetylated HMGB1 increases during cholestasis throughout the first 72 hours of injury when inflammation begins to peak [65], and is increased in human patients with cholestatic liver injury [20]. This acetylated form of HMGB1 may serve as a potent chemokine for neutrophil recruitment during cholestasis [79]. TLR expression levels increase in both hepatocytes and cholangiocytes of chronically cholestatic patients [80, 81]. Moreover, activation of TLRs on liver immune cells stimulates local NK cells to attack and kill bile ducts during primary biliary cirrhosis [82]. As blockade of TLR4 signaling via TLR4 depletion in hematopoietic derived cells [83] or the use of neutralizing antibodies to deplete HMGB-1 [84] has previously been shown to be protective against sterile inflammatory injury in the hepatic ischemia reperfusion model, further studies investigating the role of TLR signaling, HMGB1 and its acetylated form, acetyl-HMGB1, are indicated. In the mouse BDL model, endotoxin-resistant animals, which have a defect in TLR4 signaling [85], are not protected suggesting that neither gut-derived endotoxin nor other TLR4 ligands (e.g. HMGB1) are critical for the inflammatory response [18]. However, these studies are complicated by the fact that HMGB1 has additional receptors with additional

functions. Amongst these is the receptor for advanced glycation end products (RAGE) [86]. The delineation of RAGE mediated and TLR mediated effects of HMGB1 or acetylated HMGB1 are necessary to understand both in the context of cholestatic liver injury, and during general inflammation, before therapeutic options become a reality in this field, although blockade of these receptors or their ligands may serve as a therapeutic target in the future.

Osteopontin (OPN) is another recently discovered mediator of chemotaxis during cholestasis [87]. Osteopontin is a pleiotropic molecule with a role in numerous cellular activities. Secreted OPN acts as chemokine that binds integrin receptors and functions as a potent neutrophil chemoattractant [88]. Osteopontin is localized to cholangiocytes in normal liver [89]; however, chronic injury induces expression of OPN in hepatocytes [90]. Release of OPN from bile duct epithelial cells (BDECs), presumably due to the increased biliary pressure during obstructive cholestasis, mediates a substantial portion of the early immune response [87]. OPN$^{-/-}$ mice were protected against BDL-induced injury and hepatic neutrophil recruitment during cholestasis and nearly completely protected against the focal necrosis associated with bile infarcts at very early time points [87]. Activation of OPN is apparently required, as prevention of OPN cleavage by inhibition of matrix metalloproteinases in bile was protective against the early injury [87]. However, this protection was temporary, as injury returned to wild-type levels in mice by 72 hours post BDL [87]. Thus, there are likely redundancies in place to ensure neutrophil chemotaxis during cholestasis. In fact, the second wave of neutrophil chemotaxis is most likely caused by CXC chemokines [18]. These pro-inflammatory mediators are generated by hepatocytes when exposed to biliary levels of bile acids, which come from ruptured bile ducts during obstructive cholestasis [18].

Despite the strong chemotactic gradient present in the liver during BDL, it is unlikely that long term protection could be achieved through inhibition of single components of chemotaxis. Alternative options would be to inhibit upstream signaling components of cholestasis in cholangiocytes or hepatocytes to prevent activation of the inflammatory system, or to prevent the injury that occurs due to neutrophil inflammation by protecting hepatocytes against neutrophil-mediated oxidative stress through enhancement of anti-oxidant capabilities. These avenues are currently being pursued.

3.4 Innate Immune-Mediated Oxidant Stress during Cholestasis

Neutrophils execute their cytotoxicity mainly through generation of reactive oxygen species (ROS) [91]. While ROS are generated intracellularly in all cells during mitochondrial respiration, these species are largely detoxified through intracellular antioxidants. In contrast, neutrophils and other innate immune cells generate significant quantities of highly cytotoxic ROS that they use to kill hepatocytes during sterile inflammation. This requires activation of neutrophils and then extravasation into the hepatic parenchyma where release of these toxic mediators can directly damage hepatocytes [92]. Activation of neutrophils involves increased surface expression of the Mac-1 complex (CD11b/CD18), shedding of L-selectin and priming for ROS formation [93]. Neutrophil activation has been reported in response to cytokines, chemokines and complement factors [93], and also to HMGB1 [94],

and bile acids [95], which are elevated in serum during cholestasis [2, 20]. After extravasation, neutrophil adhere to target cells through CD18-ICAM-1 interactions, which triggers a long-lasting adherence-dependent oxidant stress [92]. Neutrophils produce superoxide anion through NADPH oxidase (Nox2), which then dismutates into oxygen and hydrogen peroxide [91]. The neutrophilic enzyme myeloperoxidase converts chloride anion and hydrogen peroxide into the highly toxic hypochlorous acid. While Kupffer cells are capable of producing oxidant stress, and do so in other models of sterile inflammation such as the hepatic ischemia-reperfusion model [96], the predominant oxidative stress during cholestasis likely comes from neutrophils. Kupffer cell and monocyte levels stay fairly consistent during the first two weeks of cholestasis and depletion of Kupffer cells worsens the injury due to a reduction in protective acute phase cytokines and protein production [67]. More importantly, chlorotyrosine-protein adducts are prevalent in the areas of necrosis after acute BDL injury [5, 55, 59]. These chlorotyrosine-protein adducts are indicative of an intracellular oxidant stress in hepatocytes and nearby endothelium, suggesting that neutrophil-derived hypochlorous acid must diffuse into local hepatocytes, where it causes toxicity [91]. As hypochlorous acid is a potent oxidant, it can bind and damage various components of the cell. Hypochlorous acid has been shown to form adducts with DNA and protein, and is particularly predisposed towards binding thiol groups on proteins [97], and towards forming toxic chloramines [98]. It is likely hepatocytes exposed to neutrophilic ROS are already compromised, as even low levels of toxic bile acids impair mitochondrial function without killing the cell [99]. Further exposure to ROS would then result in cell death due to aggravated mitochondrial impairment. Ablation of the mitochondrial permeability transition pore (MPTP) with NIM-811, a cyclosporine analogue, is protective against the early BDL injury, although it does not protect against later fibrosis [100]. Additionally, preservation or enhancement of intracellular antioxidants is protective against BDL. For example, kelch-like-ECH-associated-protein-1 (Keap-1) knockdown mice with a constitutively active nuclear factor E2 related factor-2 (Nrf2) are protected against BDL-induced liver injury, although some portion of this may be through enhanced choleresis [101]. More information is required in this area to assess if targeting the neutrophilic oxidant stress can prevent cholestatic liver injury. However, a general impairment of the neutrophil's cytotoxicity, which is possible using interventions against NADPH oxidase or β_2 integrins (CD18) may be effective but bears the risk of increased susceptibility to infections. Thus, strengthening of hepatocellular antioxidant systems specifically against a neutrophilic oxidant stress may be a more viable option.

4. Conclusions

Cholestasis is a complex, multi-cellular pathology involving multiple biological compartments in the liver. Modeling inflammation during cholestasis increases the complexity further as one includes additional cell types, molecular mediators and signals, and cellular stress to the pathophysiology. Studies in the past have focused largely on the role of bile acids in cholestasis; however, new data suggest the injury is far more complex. Novel roles of bile acids have recently been identified and must be explored further in appropriate rodent models of cholestasis. Future studies on the mechanisms and signaling agents involved in cholestatic liver injury should focus not only on the development of the

cholestasis itself, but the subsequent inflammation, keeping in mind that blocking inflammation is only productive when it can be done specifically. Most importantly, there is a significant gap in the amount of information available in rodent models versus human patients (Figure 3). More translational work is required in the area to discern which, if any, of these same mechanisms are relevant for the human pathophysiology.

5. Expert Opinion

Considerable efforts went into better understanding cell death processes associated with cholestasis. The mainline therapeutic, ursodeoxycholic acid, has been in use clinically for over two decades, and while it reduces transaminase levels in some patients, it lacks efficacy when it comes to long-term improvement [102]. Newer formulations of UDCA, including nor-ursodeoxycholic acid (nor-UDCA), may offer an improvement over classical UDCA, as nor-UDCA has shown increased efficacy in a number of murine models [103]. A majority of the current models, such as BDL, are very good models of obstructive cholestasis; however, they fail to recapitulate aspects of more complex chronic diseases. Moreover, while classical cholestasis, such as obstruction of the common bile duct, can be treated with endoscopy, new therapeutic targets are needed for successful treatment of advanced cholestatic disorders such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC), both of which prominently feature inflammation. While the therapeutic targets discussed in this review may serve as viable targets for pure cholestatic disorders, better models of both PSC and PBC are needed where these targets can be further validated. More effort should be placed towards understanding the mechanisms behind these clinically relevant disease states, and currently understood targets should be re-evaluated in the context of more advanced models and when possible, in patients with PSC and PBC.

One concept this review attempts to address is the idea of differential susceptibility to bile acids between species, and how inherent bile acid production and accumulation is likely to be partially responsible for this difference. While we have previously argued for a limited role for bile acid toxicity during cholestatic liver injury, recent data from primary human hepatocytes suggests human hepatocytes are acutely susceptible to bile acid-induced liver injury, but only in the context of biliary concentrations of bile acids [20]. These data are supported by the fact that human hepatocytes do not undergo toxicity until bile acid concentrations of 500μM or more are added for an extended period [19, 20]. Hepatocytes may be exposed to these concentrations when local biliary tracts rupture due to the increased pressure associated with cholestasis [7, 20, 30]. This is consistent with the presence of extra-biliary bile in patients with acute extrahepatic cholestasis [20]; although, patients with chronic intrahepatic cholestasis do not always recapitulate this facet of cholestatic liver injury. This may be due to chronic compensation and subsequent alteration of relevant transporter levels, and thus inflammation may play a greater role in these disease states. One important caveat is the idea of *intracellular* bile acid accumulation and what role it plays in bile acid-induced toxicity, especially in the context of chronic exposure to elevated bile acid levels in patients. Measurements of bile acids from liver tissue do not reflect measurements in hepatocytes themselves, as any biopsy would also include multiple other cell types including blood vessels, biliary tracts, and other hepatic architecture. Experiments measuring retention of bile acids in isolated hepatocytes after exposure to mixed or single

bile acid concentrations may yield novel and important information about the kinetics of acute or chronic bile acid exposure, and how this effects toxicity. Isolated primary human hepatocytes and HepaRG cells will likely make good models for this as they retain transporter activity for longer periods *in vitro* [20, 104, 105].

Recent data from our group and others suggest a re-evaluation of the levels of bile acids used during *in vitro* experimentation [2, 3, 14, 20]. For example, studies have repeatedly demonstrated that unconjugated levels of bile acids rarely accumulate to micromolar concentrations *in vivo*. Chenodeoxycholic acid is a potent activator of FXR; however, levels of unconjugated chenodeoxycholic acid in serum are typically two orders of magnitude below concentrations routinely used *in vitro*. As hepatocytes are unaccustomed to these high concentrations of bile acids, this may yield artifacts specific to the *in vitro* system. Potentially the best example of this is the profound susceptibility of rat hepatocytes to bile acid-induced apoptosis as compared to either mouse or human hepatocytes.

It should be noted that while this review focused on bile acid signaling in hepatocytes and the subsequent inflammatory response, bile acid signaling in cholangiocytes may be another potential therapeutic area. Recently, it was established that cholangiocytes protect themselves against bile acid induced injury through a protective glycocalyx featuring an "umbrella" of bicarbonate secretion that lowers local pH values and reduces bile acid uptake through the anion exchange 1 transporter [106]. This new therapeutic area holds a great deal of potential as drugs specifically targeted towards the bile may be especially effective at preventing the adverse reactions associated with bile acid-induced cholangiocyte damage.

Finally, increased effort needs to be placed on understanding the complex role of inflammation during cholestasis. A number of different DAMPS and cytokines likely have integral roles in the pathogenesis and recruitment of inflammatory cells of cholestatic disorders including molecules such as HMGB-1 [20], osteopontin [87], IL-17 [46], complement C5 [107], and IL-33 [108]. Interventions against inflammation are protective in multiple models of cholestatic liver injury [5, 6, 109], and a number of different inflammatory populations have been indicated as potentially pathogenic in multiple models of cholestasis [5, 6, 55, 109, 110]. Even so, limited resources have been placed on understanding the role of specific mediators of inflammation in the context of advanced cholestatic diseases such as PSC and PBC, or in childhood major cholestatic disorders such as biliary atresia. More studies designed at understanding the unique role of individual inflammatory mediators, both cellular macromolecules and inflammatory cell themselves, is needed to progress in the understanding of this field.

Acknowledgments

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Highlights

1. Cholestasis results in elevated bile acid levels in multiple tissue compartments.

2. Hydrophobic bile acids may directly cause injury to hepatocytes and other cells at biliary (millimolar) concentrations; this effect is species-dependent.

3. Neutrophil recruitment and neutrophil mediated liver injury is common sequelae in cholestatic liver injury.

4. Bile acids themselves may be pro-inflammatory molecules at high concentrations.

5. A number of different therapeutic targets including nuclear receptors, G-protein coupled cell surface receptors and more have been identified that may alter bile acid induced signaling.
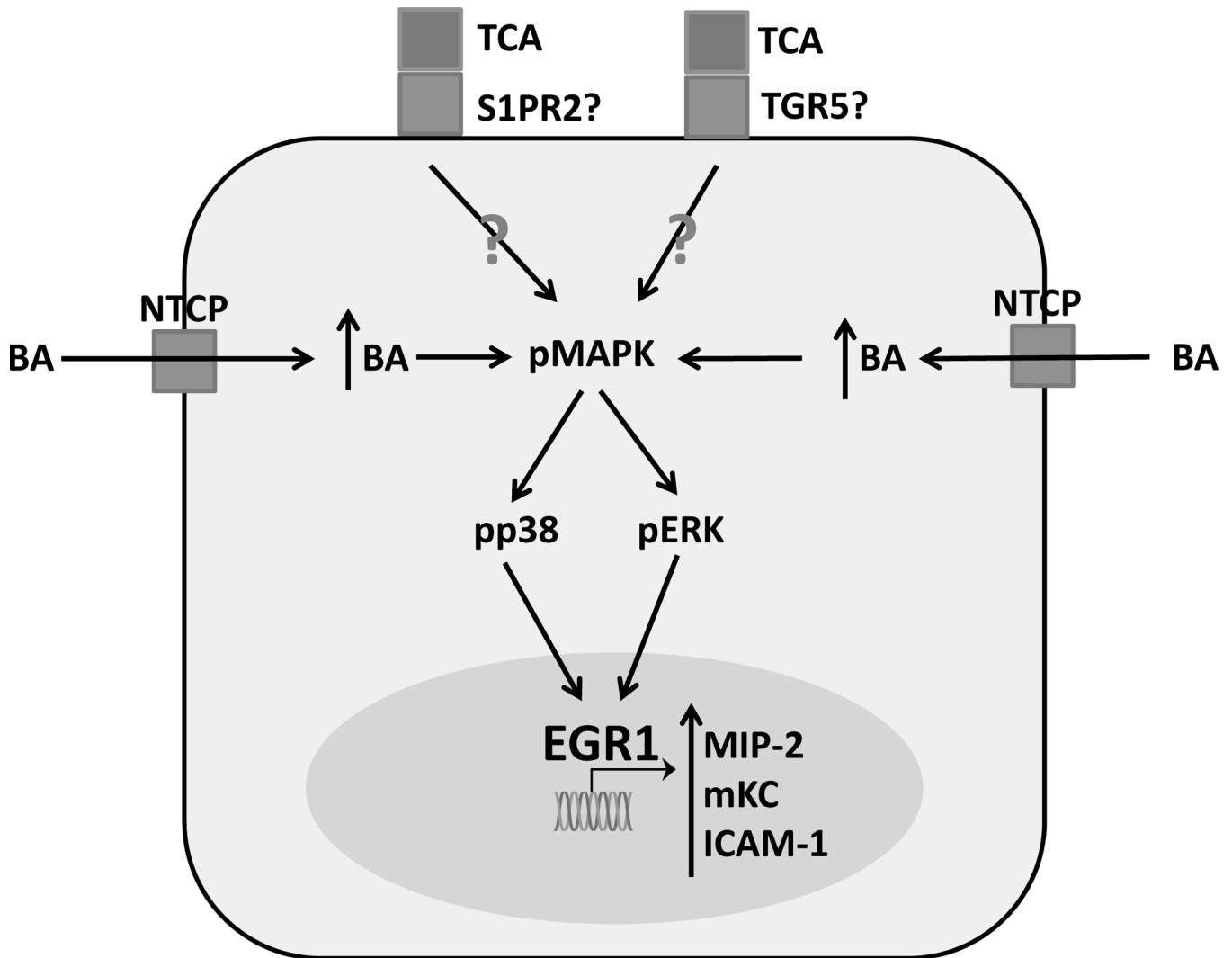
Figure 1.
Bile acid signaling in murine hepatocytes. Cultured hepatocytes exposed to conjugated bile acids such as taurocholic acid active a signaling pathway that results in dramatic increases in expression of pro-inflammatory mediators. TCA – taurocholic acid, S1PR2 – sphingosine 1 phosphate receptor 2, GPBAR-1 – G-protein bile acid coupled receptor-1, BA – bile acid, NTCP – sodium taurocholate co-transporting polypeptide, pMAPK – phosphorylated mitogen activated protein kinase, pp38 – phosphorylated protein-38, pERK – phosphorylated extracellular signal regulated kinase, Egr1 – early growth response factor 1, MIP-2 – macrophage inflammatory protein 2, mKC – mouse keratinocyte factor, ICAM-1 – intercellular adhesion molecule-1, OATP – organic anion transporting polypeptide, TGR5/ GPBAR-1 – G-protein coupled bile acid receptor
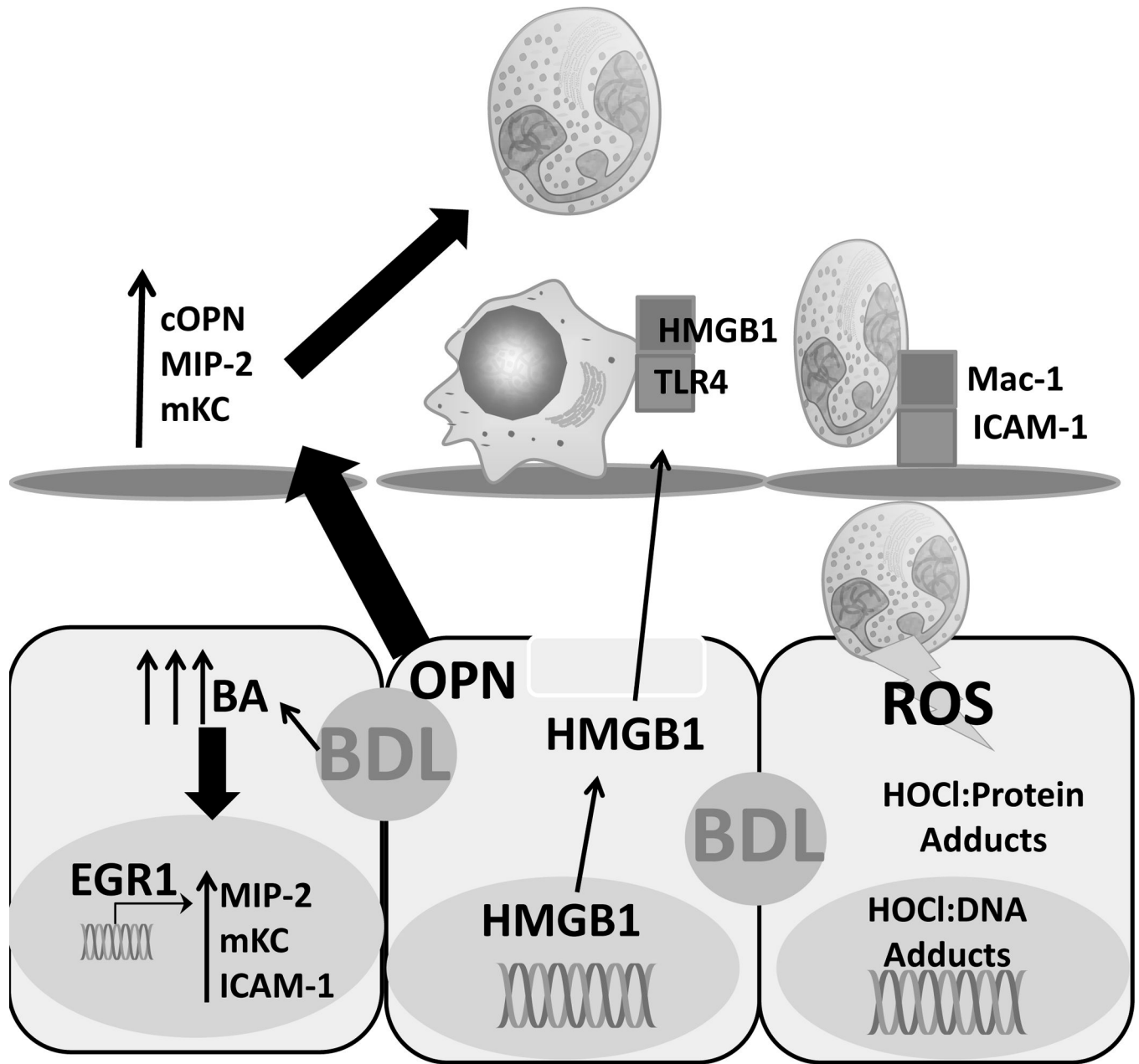
Figure 2.
Inflammation after surgically induced cholestasis. After bile duct ligation (BDL), numerous cytokines, including osteopontin present in bile duct epithelial cells, are produced that actively recruit cytotoxic neutrophils. These neutrophil extravasate through the sinusoidal endothelial cell layer and release ROS that actively kill damaged, but alive hepatocytes. BA – bile acid, MIP-2 – macrophage inflammatory protein 2, mKC – mouse keratinocyte factor, ICAM-1 – intercellular adhesion molecule-1, Egr1 – early growth response factor 1, BDL – bile duct ligation, OPN – osteopontin, cOPN – cleaved ostepontin, HMGB1 – high mobility group box-1, Mac-1 – macrophage 1 antigen (CD11b/CD18), TLR-4 – toll-like receptor 4, HOCl – hypochlorous acid, ROS – reactive oxygen species
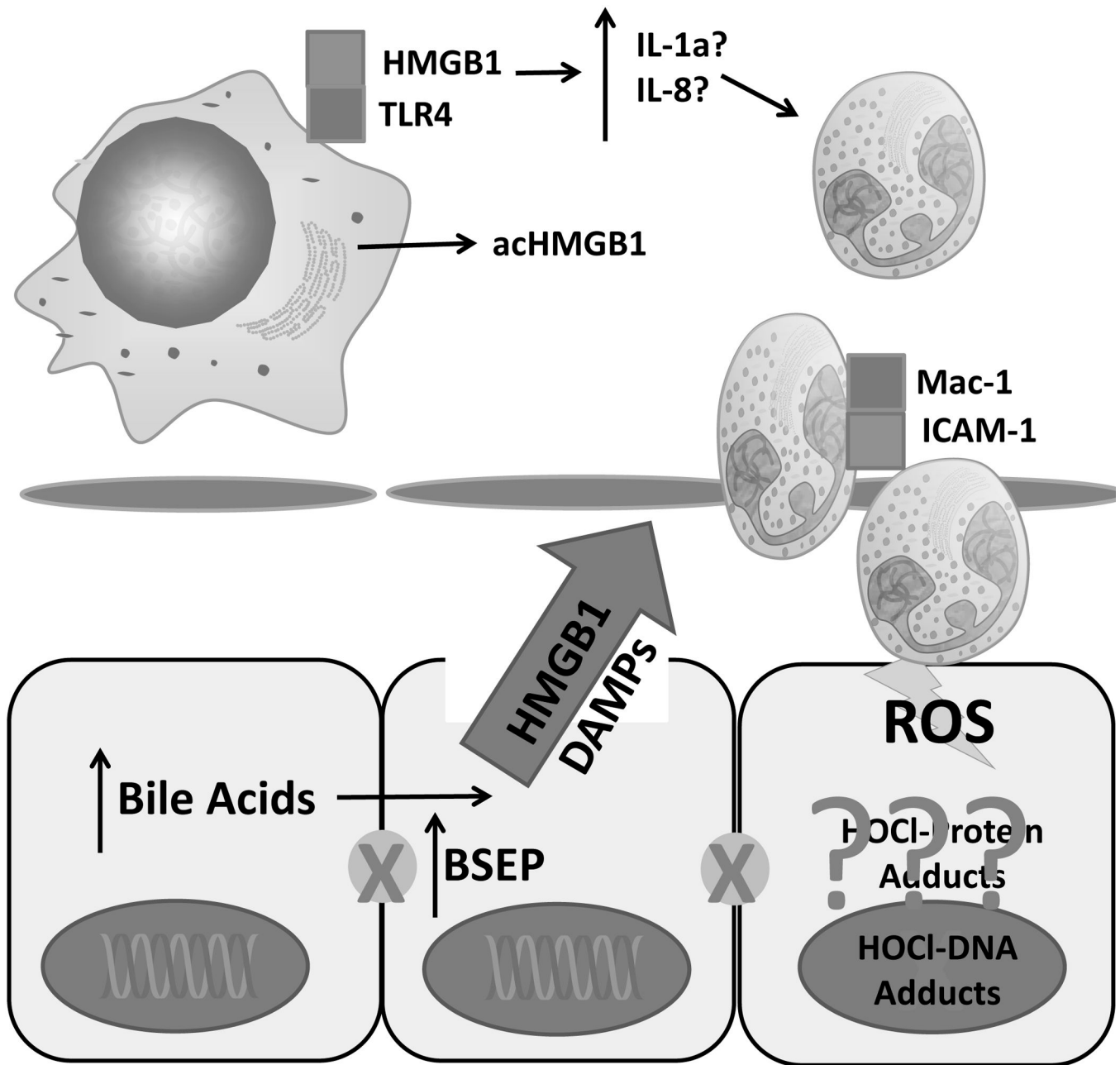
Figure 3.
Model for bile acid-induced necrosis in man. Increases in cellular bile acid levels leads directly to cell death when exposed to biliary bile acid concentrations. This results in release of damage associated molecular patterns such as HMGB1 and potentially other DAMPs. Current research is attempting to determine if the subsequent recruitment of neutrophils is mediated by specific cytokines, and if neutrophils worsen injury after cholestasis. BA – bile acid, HMGB1 – high mobility group box-1, DAMP – damage associated molecular pattern, acHMGB1 – acetylated HMGB1, TLR-4, toll-like receptor 4, IL-1α – interleukin 1α, IL-8 -

interleukin-8, ROS – reactive oxygen species, HOCl – hypochlorous acid, Mac-1-macrophage 1 antigen (CD11b/CD18), ICAM-1 – intercellular adhesion molecule 1