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THE CONTRIBUTION OF INNATE IMMUNE CELLS TO THE PATHOPHYSIOLOGY OF ACUTE STERILE INFLAMMATION-EVIDENCE FROM PRECLINICAL ANIMAL MODELS

Nardeen Eldafashi, Shaimaa Waaz, Maiiada Hassan Nazmy, Moustafa Fathy*

Department of Biochemistry, Faculty of Pharmacy, Minia University, Minia 61519, Egypt

One of the most critical factors for managing patients with liver disease is inflammation. The liver mediates numerous functions in the innate immunity, these functions predispose the liver to immunity-mediated liver injury if this inflammation is not checked. Models of sterile inflammation-related liver injury have witnessed significant attention during the last 25 years. Understanding the pathophysiology of acute sterile inflammation provides a clear vision about which mechanism is involved in this process. Many studies focused on developing and testing new therapeutic targets on different preclinical models of acute sterile inflammation with an ultimate goal of improving patient outcomes. In this review, we discuss the pathophysiology and the most common in vivo models of sterile inflammation. We then explain the most common innate immune cells that contribute to the development and progression of the disease. Finally, we discuss the interaction between these immune cells and the surrounding "niche" that favors their recruitment to the diseased livers

Keywords: *Sterile inflammation, Neutrophils, Macrophages, Acetaminophen induced liver injury, obstructive cholestasis, hepatic ischemic reperfusion injury and pro-inflammatory cytokines*

INTRODUCTION

The most common preclinical models of acute sterile inflammation involve Hepatic Ischemic Reperfusion Injury (HIRI), Acetaminophen overdose and Bile Duct Ligation (BDL). These models were carefully designed to represent the relevant human diseases to test different diagnostic and therapeutic strategies. Therefore, these models are the "golden standard" that improve the clinical outcomes of the affected patients. Clinically, HIRI is mediated by the initial storage of the graft at low temperature during transportation (cold ischemia) followed by introduction to the recipient's body (warm ischemia) and can lead to primary graft dysfunction. Some cases may develop multi-organ dysfunction syndrome (MODS) or systemic inflammatory response syndrome (SIRS)[1]. These syndromes are associated with high morbidity and mortality. A current national shortage of donor organ supply increases the importance of HIRI[2]. Thus, HIRI has become the subject of much research to study pathophysiology and find the best therapeutic target to attenuate HIRI during liver transplantation. Uncontrolled administration of Acetaminophen can also lead to acute liver failure and, if not appropriately managed, death. Initiated by gall stones or tumors, mechanical blockade of the liver duct system elicits a cascade of inflammatory process known as obstructive cholestasis.

The common link between these three clinical disorders is initiation of an inflammatory response in the absence of infectious microbes or pathogens, hence, these conditions are categorized as "acute sterile inflammation". Progression of this type of inflammation can lead to end-stage liver diseases and eventually death. It is therefore

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*Corresponding author: Moustafa Fathy, E-mail: mostafa_fathe@minia.edu.eg

important to understand the full pathophysiology of acute sterile inflammation and to mine the literature that explains the involvement of different immune cell subsets in disease progression. This will help identify novel therapeutic targets to increase patients' survival.

General pathophysiology of sterile inflammation

Sterile inflammation mediates the release of Damage-Associated Molecular Patterns (DAMPs)

Unlike infectious inflammatory responses which are driven by the engulfment of infectious species, sterile inflammatory response is initiated via DAMPs released from injured, dying or necrotic epithelial cells[3-6]. Necrosis releases the damaged cellular content into the extracellular matrix to mediate downstream deregulated receptor activation and enzyme-substrate interaction accelerating and boosting the subsequent inflammatory response. Besides, necrosis may also potentiate inflammation by modifying the extracellular matrix components that act as scaffolds and gradients for many chemo-attractants and interleukins[7].

Activation of liver-resident hepatic Kupffer cells (KCs)

Hepatic KCs are endogenous macrophage populations that reside in the sinusoidal space of liver vasculature[8,9]. The release of DAMPs mediates the polarization of KCs through the activation of a plethora of receptors like Toll-Like Receptors (TLRs), purinergic receptors, the hyaluronan receptor CD44 and the receptor for advanced glycation end products (RAGE) which are activated through binding to high mobility group box-1 protein (HMGB1). This receptor-mediated activation of KCs induces the release of several cytokines and chemokines to activate the Nuclear Factor-kappa B (NF-κB) pathway.

NF-κB consists of proteins of the Rel family that have a homologous amino acid sequence in their amino termini called the Rel homology domain[10]. This domain is involved in the dimerization, DNA binding, and the binding to the inhibitor of NF-κB (IκB) proteins. NF-κB is composed of homo- or heterodimers, however, most NF-κB molecules in the liver have a p50/p65 heterodimer complex. In physiological conditions, IκB proteins sequester NF-κB molcules in the cytoplasm and prevent their nuclear localization[11]. Two pathways are involved in the activation of NF-κB: the classical and the alternative pathways. The classical pathway results in serine phosphorylation of IκB by IκB kinase complex (IKK) and subsequent activation of ubiquitin ligase to induce IκB proteasomal degradation and the release of NF-κB subsets. The alternative NF-κB activation pathway doesn't involve IκB degradation but includes the phosphorylation of IκBα on the tyrosine residue number 42 inducing its dissociation from the NF-κB molecules. Subsequently, NF-κB subunits translocate to the nucleus and binds to the DNA to initiate the transcription of their target genes[12,13]. NF-κB activation further triggers the secretion of more proinflammatory proteins[8] priming the crosstalk between KCs and other inflammatory cells. Tumor Necrosis Factor-alpha (TNF-α)-mediated activation of NF-κB pathway regulates the expression of different adhesion molecules including P-selectin, Vascular Cell Adhesion molecule-1 (VCAM-1), and Intercellular Adhesion Molecule-1 (ICAM-1) [14] in liver sinusoidal endothelial cells (LSECs) to facilitate the recruitment of the blood immune cells to the site of injury. Activation of TLR or purinergic receptor 2X7 signaling on KCs can also initiate the activation of the inflammasome process [15,16]. The NOD-Like Receptor Protein-3 (NLRP3) inflammasome pathway represents the classical and most studied type of inflammasome in the liver. Inflammasome activation enhances the activation of Caspase-1 from procaspase-1, and further cleavage of pro-interleukin-1ß (pro-IL-1ß) to IL-1ß initiating pyroptosis that eliminate tissue remnants.

Recruitment of pro-inflammatory immune cells

In cholestatic liver injury[17], acetaminophen-induced liver injury (APAP) [18], and in HIRI[19] models, neutrophils were recruited into the liver 3-9 hours post injury. This phenotype was followed by the recruitment and activation of different monocyte/macrophage subsets[20]. This recruitment surveils the injured liver for damaged tissues and activates other tissue-

resident cells to ensure injury resolution and organ homeostasis.

Neutrophils

As in other organs, the role of neutrophils in the progression of liver injury is extensively studied [21,22]. The expression of different adhesion molecules on LSECs [23] slows down the neutrophils to help them adhere to the vasculature prior before extravasation into the parenchyma. In fact, rolling of neutrophils takes place through the interactions between different selectins and their ligands on neutrophils. The role of E-selectin and their ligands has been previously discussed in animal models where blocking selectin-mediated neutrophil recruitment promoted the hepatoprotective effect post HIRI [24]. LSEC-neutrophil crosstalk can also be initiated through the interaction between the endothelial P-selectin and P-selectin glycoprotein ligand 1 (PSGL1) on the surface of neutrophils, while endothelial ICAM-1 can interact with Mac1 [25]

Apart from adhesion molecules, neutrophil recruitment can be regulated through the release of different chemokines from hepatocytes downstream of necrosis-driven KC activation. Hepatocellular CXCL1 and CXCL2 can then interact with their cognate G protein-coupled receptors on the surface of the neutrophils initiating their migration, an effect that could be abolished by treating mice with Reparixin (an allosteric CXCR1 and CXCR2 receptor antagonist [7,26,27]. Another mechanism of neutrophil migration across LSECs is via modulating the matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [28]. In this context, mice Ly6G-positive neutrophils secreted MMP9 downstream to fibronectin-mediated activation of integrin receptor on their surface, while genetic depletion or antibody-mediated depletion of MMP9 rescued this inflammatory phenotype [29]. This finding was in line with the *in vitro* work that showed that the ability of neutrophil to migrate across fibronectin in trans-well cultures was impaired by MMP9 inhibition.

Once recruited, neutrophils enhance Reactive Oxygen Species (ROS) generation and oxidative stress-mediated damage to hepatocytes at the site of injury. Abundantly expressed by neutrophils, Myeloperoxidase (MPO) can generate chlorinating oxidants, such as hypochlorous acid (HOCl) via utilizing chloride as a co-substrate with H_2O_2. HOCL can then interact with sulfur and nitrogen-containing molecules like glutathione, cysteine and methionine. These chlorinating oxidants have one of the highest cytotoxic potentials for bacteria and living cells[30]. Activated neutrophils further enhance tissue damage by releasing neutrophil extracellular traps (NETs). NETs formation is mediated via both NADPH oxidase and protein arginine deaminase 4 (PAD4)- mediated ROS production. The role of NETs in the progression of noninfectious sterile inflammation in experimental models is, therefore, evident[22,31].

Monocytes-derived macrophages

The majority of liver macrophages are KCs in addition to other infiltrating macrophages[32,33]. Self-renewing resident macrophages originate from embryonic hematopoietic stem cells, however, under certain pathological conditions, macrophages of other origins are recruited to the tissues[32]. Abdomen-derived macrophages and bone marrow-derived monocyte macrophages are infiltered in response to infection and inflammation[34]. Macrophages can potentially differentiate into different phenotypes that show different characteristics, thus exhibiting different regulatory roles in the body's physiological and pathological activities [35]. Macrophages can polarize into two different phenotypes: classically activated M1 and alternatively activated M2[36]. Lipopolysaccharide (LPS) and interferon-γ (IFN-γ) induce M1 macrophages, whereas M2 macrophages can be activated by (IL)-4 and IL-13[37]. M1 macrophages have a pro-inflammatory function as they secrete many pro-inflammatory cytokines like inducible nitric oxide synthase (iNOS), IL-1β and TNF-α. On the other hand, M2 macrophages have anti-inflammatory potential, induce tissue remodeling, prevent parasitic infection, are involved in angiogenesis, immunity regulation, and tumor progression[38]. They mediate their function by producing anti-inflammatory factors such as transforming growth factor-β (TGF-β), IL-10 and arginase 1 (ARG1)[35].

Bidirectional association between hepatocellular injury and innate immunity in *in-vivo* models of acute sterile inflammation

APAP-induced liver injury

The initial phase of APAP-induced hepatotoxicity witnesses a significant depletion of glutathione and subsequently causes liver necrosis[39]. At this stage, oxidation by cytochrome P4502E1 leads to the formation of N-acetyl p-benzoquinone imine (NAPQI) to favor protein adduct formation and accelerate mitochondrial oxidative stress. This, in turn, generates intracellular oxidative stress and fosters liver injury via several mechanisms[40,41]. c-Jun N-terminal kinase (JNK) pathway is a mechanism which is activated, phosphorylated and subsequently translocated to the mitochondria[42]. Other contributing pathways to the injury process are SH3 domain-binding protein 5 (SAB), MAP Kinase Kinase Kinases Mixed lineage kinase 3(MLK3), and Apoptosis Signal-Regulating Kinase 1 (ASK1)[43-45]. Knockout or pharmacological inhibition of JNK, ASK1 or MLK3 showed a protective role[46]. The nuclear factor (erythroid-derived 2)-like 2 (NRF2) signaling pathway is also activated in oxidative stress and upregulates number of cytoprotective molecules. This confirms the auto protection upon further APAP dosing[47]. Knockout of NRF2 significantly worsens the liver injury[46,47], while the knockout of Kelch-like-ECH-associated protein 1 (Keap1), Which is an NRF2 binding protein, is intriguingly protective against APAP-induced liver injury[47]. Among the other pathways, B-cell lymphoma-2 (BCL-2) family proteins including BCL-2 associated X-protein (BAX) and BH3 interacting death domain agonist (BID) are also activated during oxidative stress. When these proteins are activated, they translocate to the mitochondria and start to make pores in the outer mitochondrial membrane to initiate the mitochondrial permeability transition (MPT)[48,49]. Subsequently, the receptor interacting kinase 1/receptor interacting kinase 3 (RIP1/RIP3) pathways are activated[50-52]. The current role of RIP1/RIP3 is not well understood, but they promote cytotoxicity. Collectively, MPT formation in the mitochondria is initiated by JNK translocation to the mitochondria and leads to pore formation, mitochondrial oxidative stress, pores opening, cellular dysfunction and depletion of ATP stores in the cell[42]. While there is an effective antidote to the early phase post APAP-induced liver injury. However, no effective antidote for late-stage hepatotoxicity exists. Initially, N-acetylcysteine (NAC) can restore glutathione (GSH) level and plays a major role in the detoxification of neutrophil-induced ROS via scavenging NAPQI[53]. NAC efficacy is, however, limited to early time points as subsequent APAP metabolism diminishes its hepatoprotective effects[54].

The crosstalk between hepatocytes and immune cells is evident during the second phase of injury after APAP overdose which takes place 6-12 hours following the initial phase. The mechanism is mainly due to recruited inflammatory cells which exacerbate the initial injury phase via the release of ROS and other cytotoxic compounds[42,55]. Several specific inflammatory mediators are activated during the inflammatory phase including the release of DAMPS, inflammatory cytokines and chemokines. Neutrophils are key players in this phase; however, their role is controversial. Although there was no evidence of CD11b upregulation or neutrophil activation, pretreatment with neutrophil-depleting antibodies potentiated several cytoprotective genes necessitating more efforts to elucidate this effect[18].

Obstructive cholestasis

The blockage in the bile flow from the liver to the intestine leads to "cholestasis". This blockade may occur within the internal bile duct or in the external bile duct which connects the liver to the intestine. Regardless of the obstruction site, liver injury happens as a result of severe obstructive cholestasis in both animals and humans [17,56]. BDL represents an inflammatory model of liver injury in rodents. An immediate increase in biliary pressure happens after BDL and causes rupture of the bile duct and release of bile acid into hepatocytes within 6 hours. [57,58]. Exposure of hepatocytes to accidentally high concentrations of bile acids cause necrosis, apoptosis and upregulation of several cytokines that activate KCs. Building up bile acids also induces necrosis and DAMP release to elicit the

immune response. BDL can directly affect inflammation via an early growth response factor-1 (Egr-1)-dependent signaling pathway. Neutrophil activation post BDL is documented in the areas surrounding biliary infarction with an elevated NADPH oxidase activity [59,60].

HIRI

HIRI is defined as a pathological process that consists of two cell-mediated phases: an ischemic phase which is a lack of oxygen supply to the organ, followed by a paradoxical exacerbation upon reperfusion of the liver[61]. Hepatocellular damage is initiated by warm ischemia, during liver transplantation, trauma and shock when there is insufficient blood supply to the liver[62]. However, cold ischemia is considered unique to the setting of liver transplantation and is mediated either by the LSECs or by the disruption of the microcirculation during cold storage of the organs awaiting transplantation[63]. Regardless of the type of ischemia, HIRI results in elevation of liver enzymes and graft dysfunction[64].

Pathogenesis of HIRI

Numerous cell types are involved in the pathogenesis of HIRI. Besides, several signaling pathways are activated during HIRI. Two phases have been identified, the ischemic phase which initiates cellular damage, followed by the reperfusion of the liver that exacerbates this damage. Furthermore, the reperfusion phase can be divided into early phase, which last for 2 hours after reperfusion, and late phase that can take up to 48 hours post reperfusion [65,66]. The mechanism underlying the early phase is the activation of KCs and LSECs, and the generation of ROS, whereas the late phase is mediated by neutrophil infiltration and $CD4^+$ T-lymphocytes which release proteases and other cytotoxic enzymes[67-69].

TLRs are key mediators of the innate immune response post HIRI. They are expressed on the surface of many liver cell types including KCs, dendritic cells and hepatocytes where they recognize danger molecules (DAMPs or PAMPS) to activate the immune response[70]. Following HIRI, ROS start to release HMGB1, which is a key DAMP molecule and an endogenous ligand for TLR4[71]. TLR signaling cascade activates the expression of different proinflammatory mediators including TNF-α, IL-6, and ICAM-1[72].

Proinflammatory cytokines are essential for mediating the inflammatory response. For example, IL-12 and IL-23 are upregulated as fast as 1 hour post reperfusion and disappear 4 hours later[73]. Although being characteristic features of early liver damage, neutralization of IL-12 and IL-23 results in a decrease in neutrophil infiltration and liver injury due to a decrease in the level of TNF- α and IFN-γ[74]. To understand the hepatoprotective effect of two Oxindole derivatives post HIRI, our group revealed that both Oxindole-Curcumin (Coxi) and Oxindole-Vanillin (Voxi) prophylactic treatment to a rat model of HIRI has significantly reduced the protein levels of TNF-α and IL-6 confirming their anti-inflammatory effect[22]. However, we have not identified the cellular source of TNF-α and IL-6 in our experimental settings[22].

IL-1β has an essential role in the propagation of inflammation in the early response phase after HIRI[75]. Activation of IL-1β via inflammasome pathway is one key activated pathway during HIRI. This was confirmed in our experimental settings at which Coxi and Voxi derivatives effectively attenuating pyroptosis by reducing the protein expression of NLRP3 and cleaved Caspase-1 levels and the expression of *IL-1β* in liver tissue homogenate[22]. TNF-α expression, liver inflammation, and injury after HIRI can significantly be decreased upon using IL-1β signaling antagonist[76]. TNF-α is the most important mediator in the inflammatory response to HIRI[77]. Numerous liver cells are responsible for the release of TNF-α, but KCs cells are chief secretory cells[78]. TNF-α activates hepatocytes and KCs to produce neutrophil chemoattractants like CXC chemokines. Besides, TNF-α upregulates the adhesion molecules ICAM-1, VCAM-1, and P-selectin on LSECs[79]. Neutralization of TNF-α ameliorates the hepatic injury and the inflammatory response following HIRI[79].

The role of NF-κB in HIRI is complex and cell type-dependent. In the initial phase of injury, oxidative stress and proinflammatory stimuli are responsible for NF-κB activation and the immediate increase in the expression of inflammatory mediators, cytokines,

chemokines, and adhesion molecules. In KCs, the expression of TNF-α and IL-6 are raised after HIRI following NF-κB activation[80]. In the endothelial cells, NF-κB activation elevates the expression of IL-8 and the adhesion molecules E-selectin, ICAM-1, and VCAM-1[81,82]. We proved that HIRI induced the translocation of the NF-κB subunit p-P65 to the hepatocyte nuclei; an effect that was dampened by pretreatment with Coxi and Voxi[22]. This drug-mediated pathway inhibition was associated with a reduction in *ICAM-1* and *VCAM-1*, most probably from hepatocytes or LSECs[22].

Another major immune contributor to the HIRI-induced early phase liver injury is the complement system. Complement is an immune-related system composed of small circulating proteins and has a crucial role in immune defense and inflammation against pathogens. Cellular proteins that are activated following reperfusion start to activate the complement cascade. Indeed, the presence of activated complement is detected in human liver and rodent liver after HIRI and participates in the inflammatory response[83,84]. The deficiency of C3 or the usage of C5aR antagonist results in less neutrophil recruitment and decreases the damage after HIRI[85,86]. Three distinct pathways are involved in the activation of the complement: the classical, alternative and mannose-binding lectin pathway. Improvement in microvascular perfusion, attenuation in hepatic injury and decrease in neutrophil recruitment results from the inhibition of the classical or alternative pathways[83,87,88]. Thus, complement represents one of the upstream regulators of HIRI.

Conclusion

Acute sterile inflammation is a clinical problem that affects many people globally. The contribution of different immune subsets to this phenomenon has been proved experimentally and clinically. Identification of novel therapeutic targets is essential to improve the clinical outcomes of the affected population. These new therapeutic agents should also be designed to modulate the immune microenvironment to reduce immune-related cell death, increase liver graft availability and improve the clinical picture for graft recipients.

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مساهمة الخلايا المناعية الفطرية في الفيسيولوجيا المرضية للالتهاب المعقم الحاد - أدلة من النماذج الحيوانية قبل السريرية

ناردين الدفاشي – شيماء واز – ميادة حسن نظمي – مصطفى فتحي*

قسم الكيمياء الحيوية، كلية الصيدلة ، جامعة المنيا، المنيا ٦١٥١٩ ، مصر

أحد العوامل الأكثر أهمية لإدارة المرضى الذين يعانون من أمراض الكبد هو الالتهاب. يتوسط الكبد العديد من الوظائف في المناعة الفطرية، وهذه الوظائف تعرض الكبد لإصابة الكبد بوساطة المناعة إذا لم يتم فحص هذا الالتهاب. شهدت نماذج إصابة الكبد المرتبطة بالالتهابات المعقمة دون الاصابة باي عدوي اهتمامًا كبيرًا خلال السنوات الخمس والعشرين الماضية. إن فهم الفيزيولوجيا المرضية للالتهاب المعقم الحاد يوفر رؤية واضحة حول الآلية التي تشارك في هذه العملية. ركزت العديد من الدراسات على تطوير واختبار أهداف علاجية جديدة على نماذج ما قبل السريرية المختلفة للالتهاب المعقم الحاد بهدف نهائي هو تحسين نتائج المرضى. في هذه المراجعة، نناقش الفيزيولوجيا المرضية والنماذج الأكثر شيوعًا للالتهاب المعقم في الجسم الحي. ثم نقوم بشرح الخلايا المناعية الفطرية الأكثر شيوعا والتي تساهم في تطور وتطور المرض. وأخيرًا، نناقش التفاعل بين هذه الخلايا المناعية والمكان المحيط بها الذي يفضل تجنيدها في الكبد المريضة.