EXTRACELLULAR MITOCHONDRIA RELEASED FROM LIVER ISCHEMIA REPERFUSION INJURY ACT AS ALARMINS TO EVOKE INNATE IMMUNE SYSTEM AND TRIGGER INFLAMMATION

by

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DEDICATION PAGE

Few things in life, if any, are individual accomplishments.

This thesis is dedicated to all the people that helped me get here.

Particularly,

For my wonderful husband, Chi

For my adorable daughter, Emma

For my lovely parents, Ms. Dongzhen Cai and Mr. Hengfa Hu

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ABSTRACT

Ischemia reperfusion injury (IRI) during liver transplantation enhances the immunogenicity of allografts and thus impacts overall graft outcome. This sterile inflammatory insult is known to activate innate immunity and propagate organ damage through the recognition of endogenous alarmins. The purpose of this study was to investigate the role of mitochondrial alarmins in the pathogenesis of hepatic IRI and the mechanism of immune activation by mitochondria. Mitochondria are vital cellular organelles for energy generation. However, owing to their similarity to bacteria, extracellular mitochondria function as alarmins. Recent studies revealed that mitochondria are critically involved in the initiation and progression of multiple diseases and associated with immune dysregulation. Increased levels of mtDNA and free mitochondria (FM) release were observed in both transplantation-associated and warm IR. Co-culture of mitochondrial components with hepatocytes significantly increased cell death. Mast cells (MC) and Kupffer cells (KC) are versatile immune sentinel cells known to respond to both external and internal stimuli as a first line of defense in liver. However, the response of MC and KC to mitochondria was unknown. Injection of FM into mice led to neutrophil recruitment and macrophage activation within the peritoneal cavity at 16h. MC-deficient mice had significantly reduced neutrophil responses to FM although macrophage activation was still observed. Culture of MC or macrophages with mitochondria resulted in increased production of multiple mediators. FM induced histamine release from human CBMC and mouse BMMC. This degranulation process was rapid and largely independent of extracellular calcium. Pharmacological approaches revealed that FPR1 was important for mitochondria-mediated *in vitro* degranulation and mediator production, in addition to *in vivo* sterile inflammation. FM also enhanced macrophage activation, although this was in an FPR1-independent manner. FM were potent activators of both human and mouse innate immune cells, such as MC and macrophages, *in vitro* and induced substantial MC-dependent, neutrophilic inflammation *in vivo*.

Regulation of mitochondria-mediated immune responses may be important during the initiation or progression of human disease such as acute graft failure or chronic graft versus host disease in liver transplantation, rheumatoid arthritis, or in patients with blood transfusion, which have all been reported to demonstrate enhanced FM release.

LIST OF ABBREVIATIONS USED

2-ME	2- mercaptoethanol
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate transaminase
ATP	Adenosine triphosphate
Bak	Bcl-2- homologues antagonist/killer
Bax	Bcl-2- associated protein x
Bcl-2	B-cell leukemia/lymphoma 2
BMDM	Bone marrow-derived macrophages
BMMC	Bone marrow-derived mast cells
CBMC	Cord blood derived mast cells
cDC	Conventional DC
cGAMP	Cyclic GMP-AMP dinucleotide
cGAS	Cyclic GMP-AMP synthase
COXIII	Cytochrome C oxidase subunit III
CsA	Cyclosporin A
CsH	Cyclosporin H
CXCL	Chemokine (C-X-C motif) ligand
CytB	Cytochrome B
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPI	diphenylene iodonium
EC	Endothelial cells
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Ero1	endoplasmic reticulum oxidation 1
FBS	Fetal Bovine Serum
FcRs	Fc receptors

FITC	Fluorescein isothiocyanate
FPR1	Peptide receptor 1
FPRs	Formyl peptides receptors
FSC	Forward scatter
g	Grams
GEO	Genome Expression Omnibus
GPCR	G protein coupled receptors
h	Hours
H&E	Haematoxylin-eosin
HAEC	Human aortic endothelial cells
HMGB-1	High mobility group box-1
IFN	Interferon
IHC	Immunohistochemistry
IKK	I kappa B kinase
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
Inflamm macs	Inflammatory macrophages
IR	Ischemia reperfusion
IRF	Interferon regulatory factors
IRF3	interferon regulatory factor 3
IRI	Ischemia reperfusion injuries
Isc	Ischemia
ISGs	interferon-stimulated genes
IVC	Inferior Vena Cava
IWK	Izaak Walton Killam Health Center
KC	Kupffer cells
KO	Knockout
l	Liters
LPS	Lipopolysaccharide
M1	Classically activated macrophages
M2	Alternatively activated macrophages

MAPKs	Mitogen-activated protein kinases
MC	Mast cells
M-CSF	Macrophage colony-stimulating factor
MFI	Mean fluorescence intensity
mg	Milligrams
MHCII	Major histocompatibility complex class II
MIC	Modular Incubator Chamber
min	Minutes
ml	Milliliters
MMP	Matrix metalloproteinase
MPTP	mitochondrial permeability transition pore
mROS	Mitochondrial reactive oxygen species
mtDNA	Mitochondrial DNA
MTD	Mitochondrial lysate, named mitochondrial DAMPs
MTT	Colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2H-tetrazolium bromide
MyD88	Myeloid differentiation primary-response protein 88
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NASH	Non-alcoholic steatohepatitis
NCBI	National Center for Biotechnology Information
NET	Neutrophil Extracellular Traps
NFPs	N-formyl peptides
NFκB	Nuclear factor kappa B
NGS	Normal goat serum
NHBD	Non-heart-beating donors
NK	Natural killer cells
NKT	Natural killer T cells
NLR	Nod-like receptor family
NLRP3	NLR pyrin domain containing 3
NO	Nitric oxide
NPC	Nonparenchymal cells

PAMPs	Pathogen-associated molecular patterns
PCC	Peritoneal cells
pDC	Plasmacytoid DC
PI3K	Phosphoinositide-3 kinase
PRRs	Pattern recognition receptor
Rep	Reperfusion
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SD	Standard deviation
SEC	Sinusoidal endothelial cells
SEM	Standard error of the mean
SGs	Secretory granules
SIRS	systemic inflammatory response syndrome
sPLA2	secretory phospholipase A2
SSC	Side scatter
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TDC	Tolerogenic DC
TEM	Transmission Electron Microscopy
TFAM	Mitochondria transcription factor A
TLR9	Toll-like receptor 9
TLR	Toll like receptors
TMRM	Tetramethylrhodamine methyl ester
TNF	Tumor necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
ug	Microgram
US	United States
UW	University of Wisconsin
Wsh	Mast cell-deficient C57BL/6-Kit$^{W\text{-}sh}$ mice
WT	Wild type

α-GalCer	α-galactosylceramide
ΔΨm	Mitochondrial membrane potential

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CHAPTER 1 INTRODUCTION

1.1 Liver transplantation

Organ transplantation is currently an effective therapy for patients with many end-stage organ diseases. However, organ transplantation required substantial research and development before it was successfully introduced into clinical settings worldwide. Graft and patient survival rates were initially not very high, but with the discovery in the 1980's of cyclosporin (CsA) and other, more recently introduced, immunosuppressive agents that inhibit graft rejection, the outcome of clinical organ transplantation has dramatically improved [1]. Furthermore, due to more refined surgical techniques and improved post-operative care, survival rates almost doubled, resulting in one-year graft survival of 80% to 90% in many settings. The first attempt to transplant a human liver was performed by Thomas Starzl in 1963 [2]. In 2017, there were 464 liver transplants performed in Canada. In the same year, the reported 1, 3, 5, and 10-year survival rates for recipients transplanted in 2016, 2014, 2012, and 2007 were 93.3%, 87.6% 87.7%, and 74.2% respectively [3]. The statistics from the United States (US) are even more striking, where 8082 liver transplants were performed in 2017 [4]. Survival rates in the US are similar, with reported rates in 2016 of 90.4%, 83.7%, 75.9% and 57.0% at 1, 3, 5, and 10 years for adult transplant, respectively [4]. Due to major achievements in liver transplantation, the inclusion criteria for potential liver transplant recipients have been broadened, resulting in a persistent problem of donor organ shortages and the waiting list has grown progressively over the past 20 years. In Canada, at the end of 2017, there were 321 patients listed as waiting for liver transplantation with an additional 64 people having died waiting for an organ, representing a mortality rate of 17% [3]. In the US, waitlist candidates have continued to

increase over the past 5 years and the overall deceased donor transplant rate was 51.4 per 100 waitlisted [4]. The ongoing donor organ shortage has led to increased use of extended criteria donor organs, i.e. those from older or steatotic donors, or from donors after cardiocirculatory death, as well as organs that have been subjected to prolonged periods of cold ischemia [5].

1.1.1. Extended criteria organs

To date, the majority of donor livers that are used for transplantation originate from brain dead cadaveric donors. In this type of donor, blood circulation is intact, while brain death has been determined. Since blood flow to the liver is still functional, these livers are normally of good quality with high transplant success rate after preservation and transplantation. However, even with refined surgical techniques and perioperative management, the waitlist for liver transplant is still increasing. This underlines the need for additional sources of organ donors. Alternatively, improvements in donor preparation and organ preservation allow sub-optimal donor groups, such as marginal donors and non-heart beating donors (NHBD), into the liver donor pool. Livers from marginal donors may be of less high quality due to various causes including age, hypotension, hepatic steatosis, or prolonged ischemic time due to procurement complications [6, 7]. NHBD are cadaveric donors in which the hearts have stopped beating at the time of procurement. A consequence of using organs from NHBD is the period of warm ischemia post cardiac arrest, which has been demonstrated to increase cell death in donor livers [8-10]. Adding these donor organs to the transplantation pool would significantly decrease the number of patients on the waitlist, however, these extended criteria organs are more susceptible to liver ischemia reperfusion injury (IRI) [10-16].

1.1.2. Ischemia reperfusion injury (IRI)

IRI is a phenomenon whereby cellular damage in a hypoxic organ is exacerbated following the restoration of oxygen to this tissue [17]. Cellular-basis IRI can be divided into two steps: first, cells are subjected to combined oxygen-nutrition deprivation; second cells are exposed to rapid re-oxygenation. The liver, an organ with high energy requirements, is highly dependent on oxygen supply and susceptible to hypoxic or anoxic conditions [18]. Ischemia and reperfusion (IR), which involves complex inflammatory pathways, is a significant cause of liver damage and an important factor in a variety of diseases, injuries, and procedures that include, but are not limited to, liver transplant and trauma [19]. During liver transplantation, the liver undergoes a period of cold ischemia when the organ is retrieved from the donor and is stored under hypothermic conditions (termed cold ischemia). Following this, the liver is exposed to warm ischemia, when it is removed from cold storage, but before it is reperfused in the recipient. IRI in the context of liver transplantation is thus a combination of cold and warm ischemia followed by reperfusion (transplantation-associated ischemia reperfusion) [17]. Isolated warm IRI occurs during elective liver surgery, trauma and incidental events such as hypovolemic shock [20]. The pathophysiology of liver IRI includes direct cellular damage, such as hepatocyte necrosis and apoptosis. This is triggered by the ischemic insult and delayed organ dysfunction related to activation of the innate immune system, as well as the propagation of the inflammatory responses [21-25]. The ongoing donor organ shortage has led to increased use of extended criteria donor organs, e.g. those from older or steatotic donors, or from donors after cardiocirculatory death, as well as organs that have been subjected to prolonged periods of cold ischemia. Unfortunately, these organs are more

susceptible to IRI [10-16]. In liver transplantation, IRI is the major contributor to post-transplant liver graft dysfunction, patient morbidity, and mortality [26-29]. To date, no specific treatment is available to prevent or reduce hepatic IRI and current management is based on supportive care. An improved understanding of the mechanisms underlying hepatic IRI could lead to more effective therapeutic interventions to minimize liver dysfunction and improve patient survival.

Hepatic IR is a biphasic process which includes massive necrotic and apoptotic cell death responses that are initiated on the reperfusion, along with an often unsuccessful attempt of cells to re-establish homeostasis [30, 31]. Previous studies suggested that the hepatotoxic effects of IRI originate from a complex crosstalk between hepatocytes, tissue-resident immune cells and immune cells that are recruited to the site of injury [30, 32-35]. In this setting, a key contributor to the induced inflammatory response in IRI is the production of endogenous 'danger signals' (alarmins), which are released from damaged tissues or stressed hepatocytes, as discussed below.

1.2 IRI triggers release of endogenous danger signals

Innate immunity is critical to the process of solid organ transplant rejection [36, 37]. Endogenous 'danger signals' known as alarmins or damage-associated molecular patterns (DAMPs), generated in the organ in response to cell stress or injury, can activate innate immune cells to initiate an inflammatory response analogous to pathogen-associated molecular patterns (PAMPs) of infectious pathogens [38]. This innate immune cell activation provides a pivotal mechanism by which an organism can sense and respond to damaged self [39]. Alarmins are endogenous, constitutively expressed, chemotactic, and immune activating proteins or peptides that are released as a result of degranulation, cell

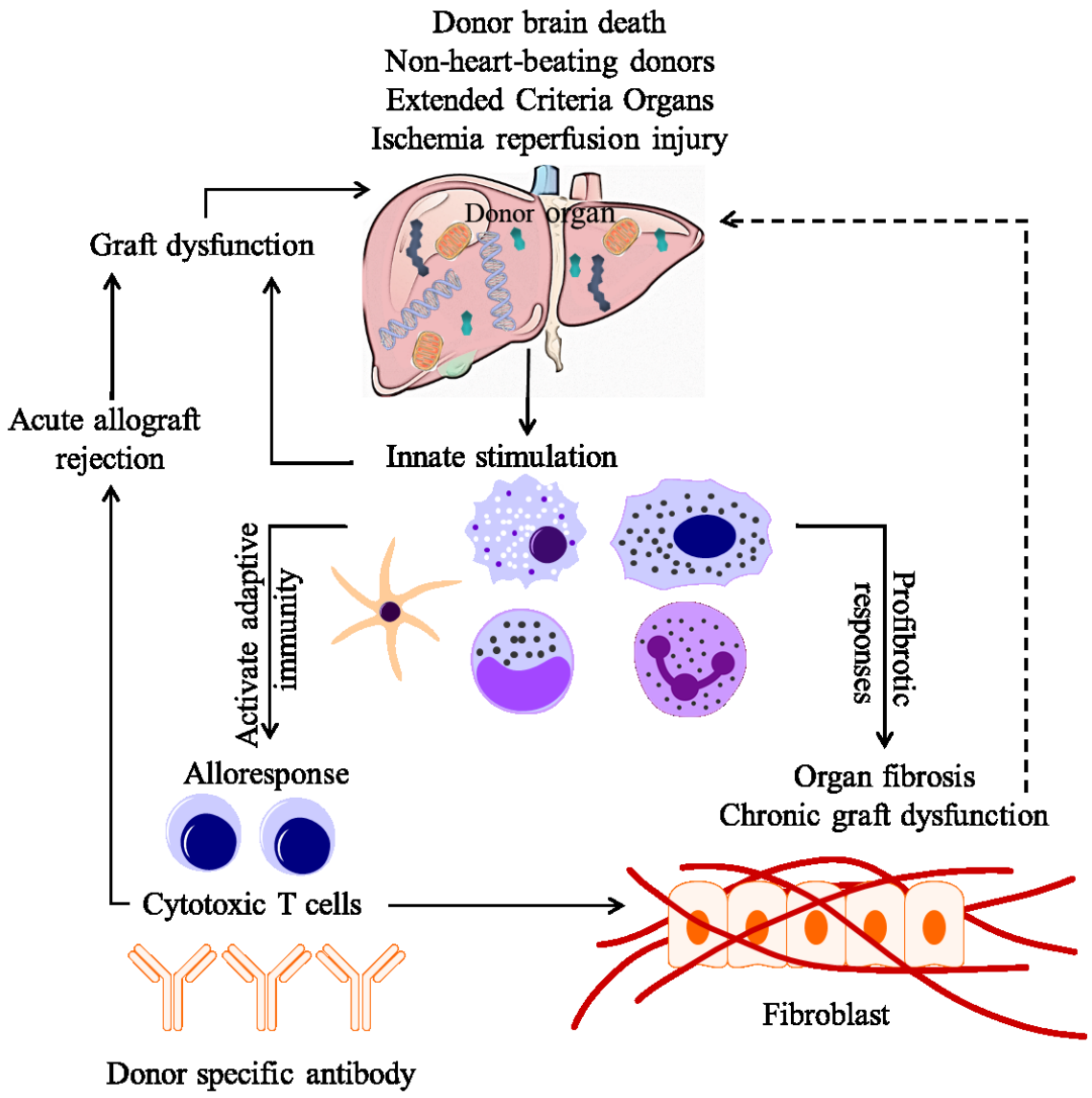
stress, injury or death, or in response to immune induction [40]. These endogenous danger signals are of particular relevance in the context of transplantation because of the injury inherent in the process of removal of an organ from one individual and later implantation into another. There is now compelling evidence that clinical events occurring across all commonly transplanted solid organs can generate endogenous danger signals (Figure 1). All transplanted organs unavoidably sustain some degree of IRI in the process of procurement and reimplantation, and IRI has been shown to directly generate alarmins from stressed tissues [36, 41-43].

Recently, several studies have described an association between the release of alarmins during ischemia and subsequent damage to cells, activation of inflammatory responses and disruption of the tissue matrix [44, 45]. Several endogenous alarmins are known to be elevated and contribute to poor outcome during liver injury. These alarmins include the high mobility group box-1 (HMGB-1) [44, 46], the cytoplasmic Ca^{2+} regulator S100 [47], the cell matrix component hyaluronic acid [48], urate [49], adenosine triphosphate (ATP) [49] and nuclear DNA [50]. Extracellular mitochondrial components are alarmins of particular interest in IRI as they are central mediators of liver injury responses [51-55].

Figure 1. Danger signals in transplantation.

Endogenous 'danger signals' or alarmins can accumulate in the donor organ during the process of donor brain death or as a result of IRI [56]. In the extracellular space, these alarmins can bind to pattern recognition receptor (PRRs), such as TLRs, or to specialized alarmins receptors to elicit an immune response by promoting the release of pro-inflammatory mediators and recruiting immune cells to infiltrate the liver. The immune cells that participate in these processes include antigen presenting cells, such as dendritic cells and macrophages, as well as T cells and neutrophils [57-59]. Evolving data suggest alarmins may also directly promote fibrotic responses and chronic inflammatory events, thereby potentially contributing, along with allo-immunity, to the development of organ fibrosis and chronic graft dysfunction [60].

Figure 1


Donor brain death
Non-heart-beating donors
Extended Criteria Organs
Ischemia reperfusion injury
Donor organ
Graft dysfunction
Acute allograft rejection
Innate stimulation
Activate adaptive immunity
Profibrotic responses
Alloresponse
Organ fibrosis
Chronic graft dysfunction
Cytotoxic T cells
Fibroblast
Donor specific antibody

1.3 Mitochondrial alarmins and their pathways

Mitochondria are the power producers of the cell and play a central role in the generation of energy from nutrient oxidation. Hepatocytes are rich in mitochondria; each hepatocyte contains about 800-2000 mitochondria, occupying about 18% of the entire cell volume [61]. Mitochondria have a unique role in the liver compared to other organs as they participate in glucose, lipid and protein metabolism. In addition to their key role in ATP synthesis, mitochondria act as crucial transducers and effectors in multiple processes including cell death signaling, oxidant signaling, innate immunity, Ca^{2+} homeostasis, fatty acid synthesis and autophagy [16]. A prime feature of hepatic IRI is mitochondrial dysfunction [62].

According to the endosymbiotic theory of mitochondrial origin, mitochondria are a descendant of the α-proteobacterium *Rickettsia prowazekii* [63]. Mitochondria still bear many morphological and biochemical features of their bacterial ancestors, including a double membrane, unique cell membrane lipids, such as cardiolipin, a circular genome containing nonmethylated CpG motifs, absence of histones, the ability to replicate independently of the nucleus, and the ability to form N-formyl peptides (NFPs) [64]. In view of their endosymbiotic origin, it is not surprising that mitochondria are also a source of alarmins leading to the activation of the innate immune system by activating pathways utilized following PAMP exposure. The liver is well positioned to undergo mitochondrial alarmin-driven inflammation due to the very larger number of mitochondria present in the metabolically active hepatocytes [65]. Previous studies have suggested that mitochondria play a critical role in immune system activation, inflammation and the pathogenesis of inflammatory diseases [66-75]. In the following sections, I will discuss recent findings

related to how mitochondrial alarmins affect immune cell trafficking and function, and how these processes can result in pathologic inflammation.

1.3.1 Mitochondrial DNA (mtDNA)

Mitochondria contain their own genetic material known as mtDNA, which represents 1% of total cellular DNA. mtDNA is organized as circular, double-stranded structure and each mtDNA molecule consists of approximately 16,000 bp. Mitochondrial DNA is packed into aggregates, called nucleoids. The most common component of nucleoids is the transcription factor A (TFAM), which has similar function to those of histones (e.g. compacting and packing DNA) [76]. A primary phenomenon of mitochondrial dysfunction is mtDNA damage [77]. Mitochondria have significant DNA repair capacity, can increase their biogenesis, and harbour mechanisms such as a ubiquitin-proteasome system and mitophagy that facilitate the removal of poorly performing mitochondria [78]. Nevertheless, mtDNA is highly sensitive to damage by reactive oxygen species (ROS) due to its proximity to the inner mitochondrial membrane where most of the ROS are produced and the lack of protection by histones and chromatin [79-81]. Therefore, mitochondrial dysfunction and mtDNA damage are often involved in diseases associated with oxidative stress, such as IRI [70].

mtDNA has emerged as an important alarmin contributing to systemic inflammatory responses [70, 82]. Current evidence regarding mtDNA-mediated inflammation is summarized in Figure 2. Similar to bacterial DNA, mtDNA contains CpG islands and is mostly unmethylated [66, 83, 84]. Bacterial DNA containing unmethylated CpG motifs is recognized by Toll-like receptor 9 (TLR9) , a member of the highly conserved PRRs known as Toll-like receptors [82, 85]. Activation of TLR9 induces nuclear

factor-kappa B (NF-κB) signaling and increases the synthesis of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-6, IL-1β [53, 86-88] and also induces p38 mitogen-activated protein kinase (MAPK) activation to promote an inflammatory response. This activation can be blocked by co-treatment of inhibitory oligodeoxynucleotides (TTAGGG) that bind CpG motifs [82]. Indeed, hepatocyte mtDNA from patients with non-alcoholic steatohepatitis (NASH) has shown substantial ability to activate TLR9. Furthermore, plasma from mice and patients with NASH contains increased levels of mtDNA and intact free mitochondria (FM), which results in a greater activation of the TLR9 pathway and a skewed pro-inflammatory response [89, 90]. The systemic distribution of mtDNA with the ability to activate TLR9 may have consequences for inflammation regulation systemically, in addition to contributing to local hepatic inflammation.

With the observation that mtDNA released into the cytoplasm is a key activator of the inflammasome, an unexpected new function for this organelle in controlling the innate immune response is now emerging [91-93]. The inflammasome complexes function as molecular scaffolds that recruit and activate caspase precursors, leading to the release of pro-inflammatory cytokines IL-1β and IL-18 [94]. The NOD-like receptor family (NLR) pyrin domain containing 3 (NLRP3 also known as NALP3) inflammasome is the best-characterized thus far [95] and can be activated by a broad range of stimuli [96]. Diverse stimuli, including mitochondrial damage, have been shown to provoke the NLRP3 inflammasome during infection and metabolic diseases [97]. Bacterial and viral RNA can also activate the NLRP3 inflammasome [98]. Two recent studies have shown that mtDNA released in the cytoplasm plays a key role in provoking the NLRP3 inflammasome [92]. In

2011, Nakahira *et al* . suggested that ATP-mediated mtDNA release depends on the NLRP3 inflammasome and mitochondrial reactive oxygen species (mROS) [92]; thus mtDNA amplifies inflammasome activation after the initial trigger. In 2012, further study showed that mitochondrial death is a prerequisite for the release of oxidized mtDNA into the cytoplasm [91]. Oxidized mtDNA was observed to directly bind to and activate NLRP3, suggesting that mtDNA can initiate the activation of this pathway. Further supporting a direct role for mtDNA in mediating inflammasome pathways, cells lacking mtDNA (generated by ethidium bromide treatment) were unable to provoke NLRP3 inflammasome activation [92]. Interestingly, NLRP3 is also required for the release of mtDNA during inflammasome activation [92]. These data suggest that mtDNA may act in a positive feedback loop to potentiate NLRP3 inflammasome activation. An accumulating body of evidence indicates that inflammation in liver IRI and murine allograft model is mediated through the inflammasome [99-102]. Evidence for mtDNA release after IRI is beginning to emerge as well [53, 70]. Through these mechanisms, mtDNA may provoke inflammasome activation in the hepatic IRI model and might further play a role in liver organ transplantation. However, there are additional key issues that remain to be resolved. For example, it is unclear whether the release of mtDNA into the circulation following tissue injury during liver IRI after organ transplantation is similarly able to activate inflammasome signaling by recruited phagocytes, thereby contributing to an ongoing inflammatory response.

In addition to activating with TLR9 and the NLRP3 inflammasome, mtDNA also trigger stimulator of interferon genes (STING) signaling. A proposed model for mtDNA activation of the cGAS-cGAMP-STING pathway is summarized in Figure 3. A series of

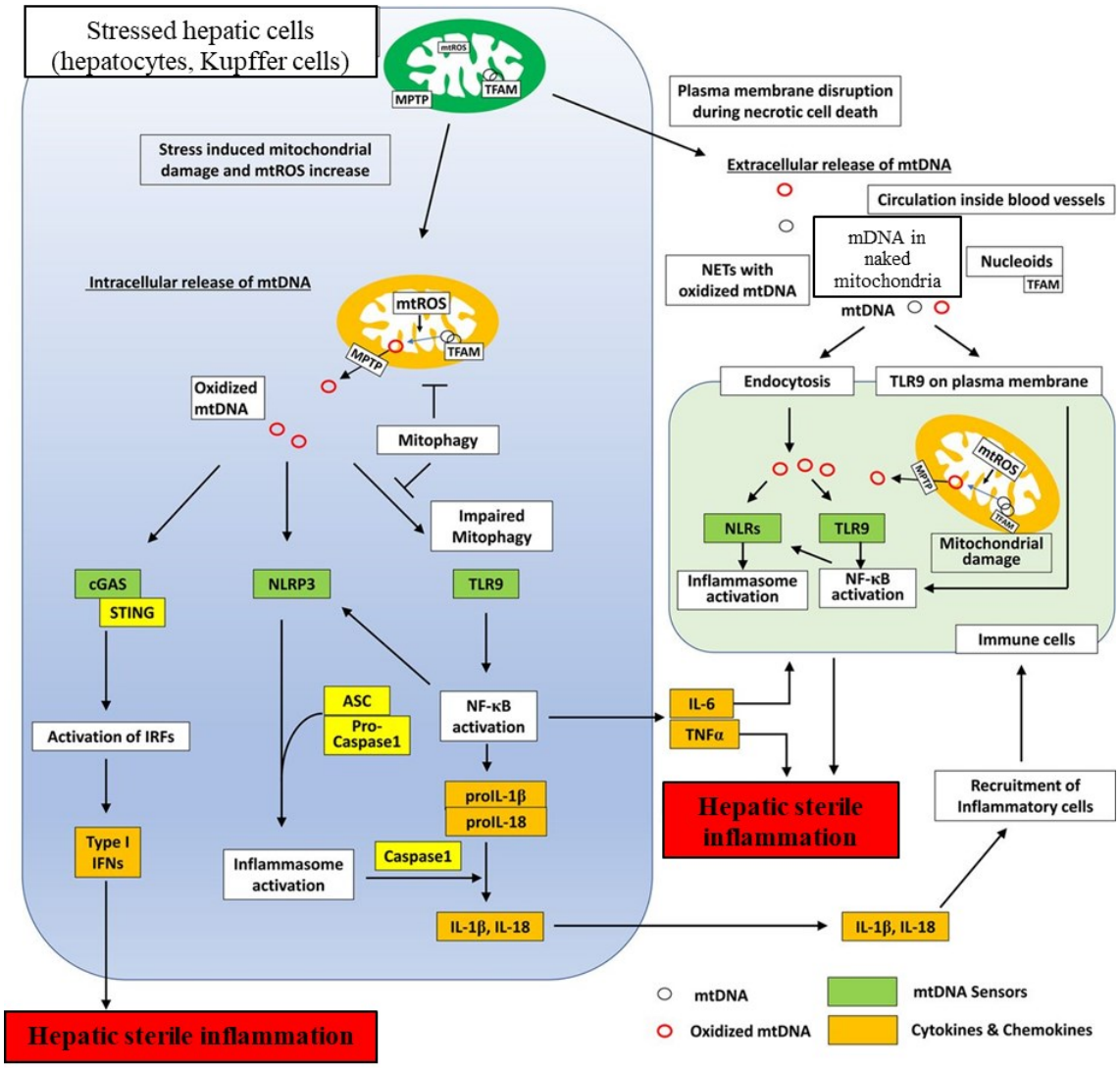
studies from the Chen laboratory have clearly elucidated the process of mtDNA sensing by cGAS-cGAMP STING, which occurs in cytosol, rather than the nucleus [103-106]. Briefly, cytosolic DNA, either released from invading pathogen or endogenous mitochondria binds to and activates cyclic GMP-AMP synthase (cGAS) in the cytosol. This results in synthesis of its second messenger cyclic GMP-AMP dinucleotide (cGAMP), which subsequently activates the endoplasmic reticulum (ER) membrane protein - STING. STING then translocates from the ER to the Golgi apparatus to reach the perinuclear endosomes before assembling with TANK-binding kinase 1 (TBK1). TBK1 subsequently phosphorylates and activates interferon regulatory factor 3 (IRF3), which results in the transcription of type I interferon (IFN) gene and other interferon-stimulated genes (ISGs) that augments viral resistance [107]. A recent study showed that TFAM depletion triggers disruption of mtDNA stability, which is characterized by nucleoid loss and enlargement. This leads to the release of fragmented mtDNA that recruits and activates peri-mitochondrial cGAS to generate the second messenger cGAMP and activate endoplasmic-reticulum-resident STING to prime anti-virus immunity [108]. Remarkably, two recent studies demonstrated that Bak- [B-cell leukemia/lymphoma 2 (Bcl-2) homologues antagonist/killer] and Bax- (Bcl-2- associated protein x) mediated mitochondrial damage, in the absence of activated downstream apoptotic caspases, induce the release of mtDNA, thereby triggering cGAS-cGAMP-STING signaling to initiate IFN production in response to viral infection [109, 110]. In addition, virus infection induces a reduction of the mtDNA packaging protein TFAM and further stresses mtDNA, characterized by nucleoid loss and mitochondria enlargement [108]. As a result, the release of fragmented mtDNA recruits and activates peri-mitochondrial cGAS to generate the second messenger cGAMP and activates STING-

TBK1-IRF3 pathway. Activation of IRF3 elevates basal gene expression of ISGs with anti-viral signaling and effector functions [108]. Whether the mtDNA released during liver IRI could be sensed by STING-IRF3, without viral infection, to induce an inflammasome-dependent pro-inflammatory response remains to be determined.

Figure 2. Mitochondrial DNA (mtDNA) modulates liver inflammation.

mtDNA binds to TFAM and is stabilized in hepatic cells. Increased mROS during stress stimulation leads to the oxidation of mtDNA and the dissociation of TFAM. Oxidized mtDNA is released via the mitochondrial permeability transition pore (MPTP). Damaged mitochondria are degraded by the autophagic process, mitophagy, and detoxified. When this process is impaired, mtDNA inside the autolysosome escape degradation and stimulate TLR9 to induce NFκB activation, which causes transcriptional activation of multiple inflammatory cytokines (IL-6, TNF, pro-IL-1β, and pro-IL-18). NFκB activation also enhances transcription of NLRP3 to prime inflammasome activation. Increased NLRP3 senses mtDNA and forms a protein complex called inflammasome with ASC and pro-caspase-1, which finally activates caspase-1 to cleave pro-IL-1β and pro-IL-18 and transform these molecules into bioactive cytokines. Secreted inflammatory cytokines from hepatic resident immune cells mediate the recruitment of inflammatory cells and hepatic sterile inflammation. cGAS senses mtDNA and activates IFN-related factors to increase transcriptional activities of type I IFNs, which contributes to hepatic inflammation. On the other hand, when the plasma membrane is disrupted by tissue damage and necrotic cell death is induced, extracellular mtDNA is released and circulates inside vessels as cell-free mtDNA. In the serum, mtDNA is observed within exosomes, as TFAM-bound forms (nucleoids), inside neutrophil extracellular traps (NETs), or in naked mitochondria organelle. mtDNA enters the endocytic pathway by endocytosis and stimulates endosomal TLR9, which leads to NFκB activation and inflammasome formation. These processes are involved in the development of hepatic sterile inflammation. Adapted from [111].

Figure 2


Stressed hepatic cells
(hepatocytes, Kupffer cells)
mtROS
MPTP
TFAM
Stress induced mitochondrial damage and mtROS increase
Intracellular release of mtDNA
mtROS
MPTP
TFAM
Oxidized mtDNA
Mitophagy
Impaired Mitophagy
cGAS
STING
NLRP3
TLR9
Activation of IRFs
ASC
Pro-Caspase1
NF-κB activation
Type I IFNs
Inflammasome activation
Caspase1
proIL-1β
proIL-18
IL-1β, IL-18
Hepatic sterile inflammation
Plasma membrane disruption during necrotic cell death
Extracellular release of mtDNA
Circulation inside blood vessels
mDNA in naked mitochondria
NETs with oxidized mtDNA
Nucleoids
TFAM
mtDNA
Endocytosis
TLR9 on plasma membrane
NLRs
TLR9
Mitochondrial damage
Inflammasome activation
NF-κB activation
Immune cells
IL-6
TNFα
Hepatic sterile inflammation
Recruitment of Inflammatory cells
IL-1β, IL-18
mtDNA
Oxidized mtDNA
mtDNA Sensors
Cytokines & Chemokines

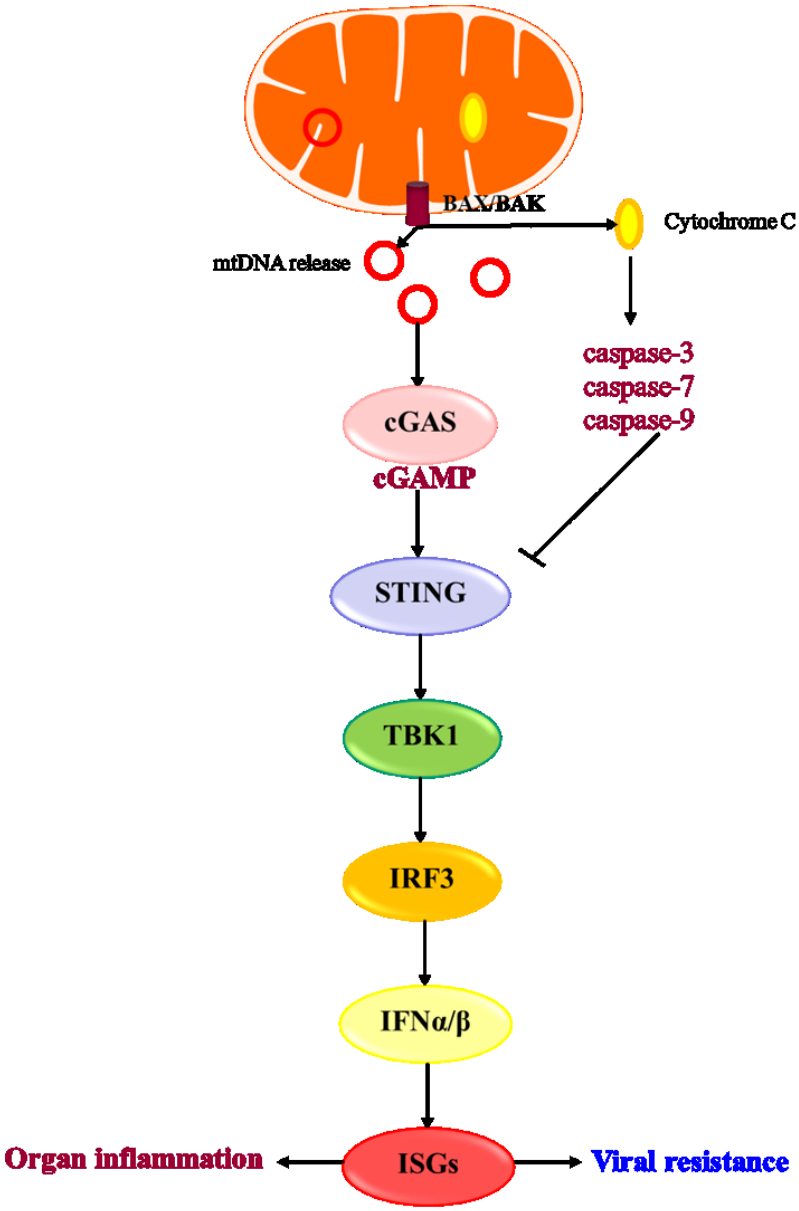


Figure 3. Overview of mitochondrial DNA induced activation of the STING pathway. In the absence of active caspases, mitochondrial outer membrane permeabilization by Bax and Bak results in the expression of type I IFNs. This induction is mediated by mitochondrial DNA-dependent activation of the cGAS/STING pathway and results in the expression of type I IFN and the establishment of a potent state of viral resistance. Adapted from [112].

1.3.2 Mitochondrial reactive oxygen species (mROS)

Oxidative stress is generally defined as an imbalance that favors the production of ROS over anti-oxidant defenses. Although sites of endogenous ROS production during normal homeostasis includes the endoplasmic reticulum oxidation 1 (Ero1)- diphenylene iodonium (DPI) oxidative folding system in the ER and the NADPH oxidase complex at the membrane, the mitochondrial electron transport chain constitutes one of the main intracellular sources of ROS [64]. During reperfusion or even , the generation of ROS and reactive nitrogen species (RNS) poses an acute threat to the viability of hepatocytes [31]. A recent study demonstrated that the generation of mitochondrial ROS led to NLRP3 inflammasome activation [113]. In 2011, Nakahira *et al* . found that autophagy limited caspase-1 activation by clearing ROS-producing mitochondria and the necessity of respiring mitochondria for NLRP3 activation [92]. In their model, culture of macrophages with LPS and ATP leads to the release of mtDNA. This release of mtDNA requires ROS formation, and is enhanced in cells lacking autophagy [92]. These observations suggested a complex model, in which NLRP3 activators (such as LPS and ATP) trigger mitochondrial ROS production which is limited by autophagic clearance of damaged mitochondria (Figure 4) [92, 113]. The consequent generation of ROS by mitochondria mediates the oxidation of HMGB-1, which promotes its immunostimulatory properties [114]. Therefore, in this model, ROS derived from mitochondria might lead to the modification of alarmins (e.g. HMGB-1) to modulate immune responses and contribute to the regulation of inflammatory disorders [64].

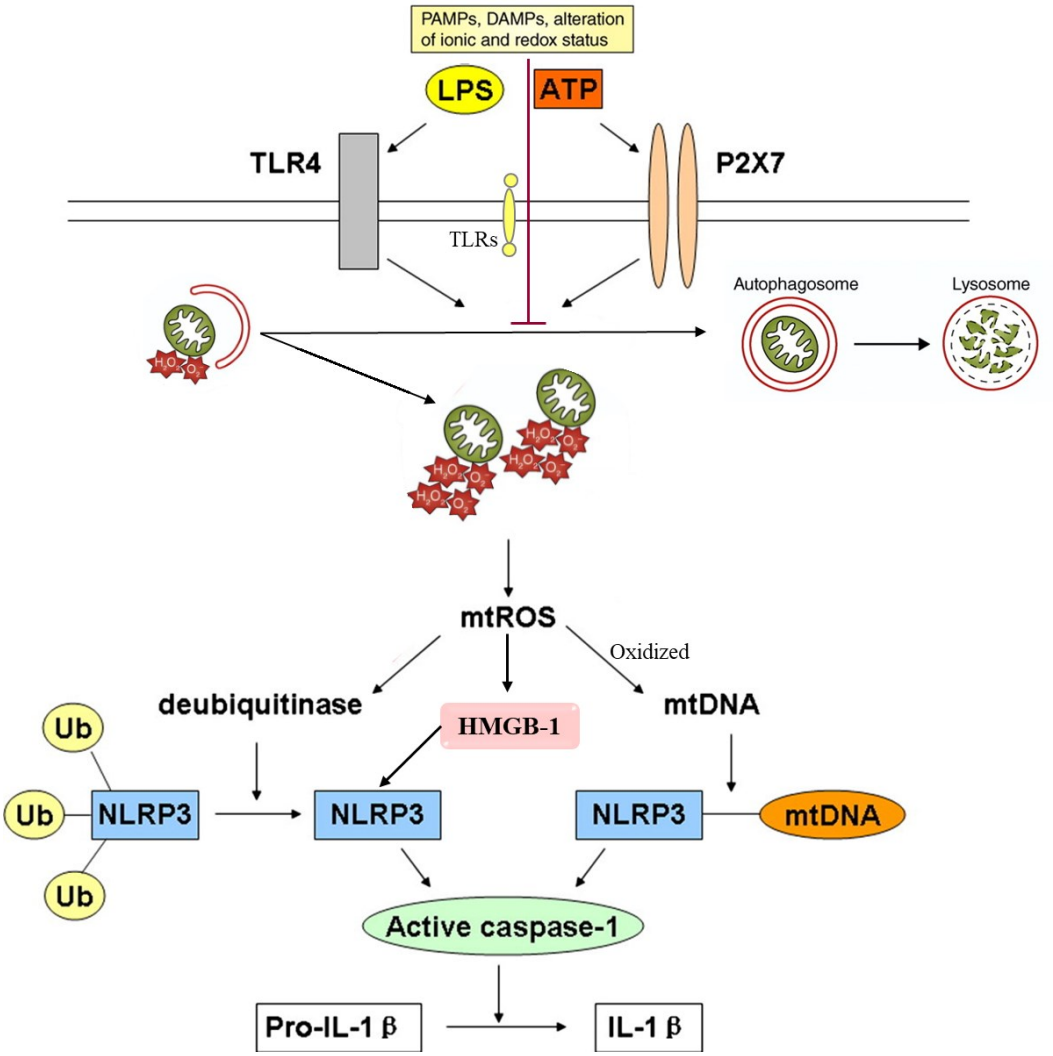


Figure 4. mROS-dependent pro-inflammatory cytokine production.

Inflammasome-dependent pro-inflammatory cytokine production requires the inhibition of autophagy and mitophagy to increase the net amount of mROS within the cell. The mechanism of this inhibition is unknown. Once mROS accumulates, it activates the NLRP3 inflammasome by mROS-mediated NLRP3 deubiquitination, the generation of oxidized mtDNA, and the modification of other DAMPs such as HMGB-1. Adapted from [115].

1.3.3 Extracellular ATP

ATP is an essential metabolic energy source in biological systems [116]. Mitochondria consume 80-90% of the oxygen in cells to support oxidative phosphorylation. This is the major and most efficient system to harvest energy from the oxidation of glucose and other fuel sources for the production of ATP [117]. Cells undergoing apoptosis or necrosis release ATP, which can also act as an alarmin (Figure 4). In this context ATP has pro-inflammatory and immunostimulatory capacity, with numerous far-reaching effects on the inflammatory response [118]. Oxygen deprivation during liver IRI induces the cells death of hepatocytes, which releases various alarmins, including ATP [118]. Studies indicate that ATP, signaling through the P_2Y_2 receptor, may induce chemotactic activity to recruit neutrophils [119, 120]. In addition, apoptotic monocytes release ATP in a caspase-dependent manner and induce their recruitment in an autocrine manner to the site of apoptosis [121]. Moreover, the presence of extracellular ATP at sterile lesions can engage the P_2Y_7 receptor and activate the NLRP3 inflammasome in macrophages and EC. This in turn results in the secretion of pro-inflammatory cytokines such as IL-1β and IL-18 [122, 123]. An elegant study has also shown that ATP released from necrotic hepatocytes, dying via accidental necrosis after local thermal injury *in vivo*, activates the NLRP3 inflammasome and alerts circulating neutrophils to adhere within liver sinusoids around foci of injury [122]. The extracellular ATP concentration is strictly maintained by CD39, a member of the ecto-nucleoside triphosphate diphosphophydrolase family that hydrolyzes ATP into adenosine monophosphate [118]. Extracellular ATP extend hepatic cold IRI associated with mouse liver transplantation, and liver resident dendritic cells (DC) could hydrolyze extracellular ATP via CD39 and attenuate pro-inflammatory cytokine

production [118]. In summary, during hypoxic conditions, the extracellular ATP is mostly a pro-inflammatory molecule to activate innate immune responses. Interestingly, liver resident immune cells, such as hepatic DC (but not limited to), are hyporesponsive to extracellular ATP because of higher expression of CD39, compared with their infiltrated counterparts.

1.3.4 Mitochondrial N-formyl peptides (NFPs)

Mitochondrial NFPs are released from damaged mitochondria at sites of tissue damage [124]. It has recently been reported that, along with mtDNA, NFPs are the major mitochondrial alarmins released in the circulation of patients with acute trauma associated systemic inflammatory response syndrome (SIRS) [82]. Similar to their bacterial homologs, mitochondrial NFPs attract neutrophils and are recognized by high-affinity G protein coupled receptors (GPCRs), formyl peptides receptors (FPRs) [122]. In IRI the interaction of FPRs with their ligands can provoke downstream responses, such as increased calcium influx, protein kinase activation, and membrane polarization [125]. It has been demonstrated that mitochondrial NFPs activate neutrophils through FPR-1 or FPR-2 and induce phosphorylation of p38, p44 and p42 mitogen-activated protein kinases (MAPKs), which leads to the release of pro-inflammatory mediators, such as matrix metalloproteinase-8 (MMP-8) and Chemokine (C-X-C motif) ligand (CXCL) 8 (CXCL8) [82, 126]. NFPs, directly and indirectly, attract neutrophils and other cell types such as thrombin-activated platelets [127]. However, whether mitochondrial NFPs play a role in immune cell infiltration to the liver during IRI has not yet been evaluated.

1.3.5 Intact free mitochondria (FM)

In addition to mitochondrial components, intact mitochondria can be released from different cellular lineages, such as neutrophils [128-130], eosinophils [131], mast cells (MC) [132], T cells [133], hepatocytes [90, 134], and platelets [135, 136] and in multiple conditions involving tissue damage [69, 90, 122, 137]. For example, intact FM are released from livers of deceased organ donors and are associated with early graft dysfunction in liver transplant recipients [134]. FM release has also been observed in chronic autoimmune disease including in the synovial fluid from patients with rheumatoid arthritis [135]. In addition, extracellular mitochondria are detected in platelet concentrates used for transfusion and are present at higher levels in those platelet preparations that induce acute reactions (febrile nonhemolytic reactions, skin manifestations, and cardiovascular events) in transfused patients [135, 138]. Recently, extracellular mitochondria have been reported to be released during traumatic brain injury. Notably, such released extracellular mitochondria were metabolically active and could serve as a source of oxidative stress that activates platelets and renders them pro-coagulant. This process was shown to contribute to the pathogenesis of traumatic brain injury induced coagulopathy and inflammation [137]. It is noteworthy that the quantification of mtDNA in the extracellular milieu is an effective measure of potential mtDNA in solution, including intact FM and microparticle containing mitochondria, all of which have different impacts. Thus, a comprehensive characterization of extracellular mitochondria as alarmins is needed to understand their impact on surrounding and infiltrated immune cells.

1.4 Liver resident cells create an inflammatory milieu during IRI

IR insult to the transplanted liver is a multifaceted process that combines elements of 'cold' and 'warm' injury. Cold ischemia, experienced during organ preservation, is dominated by damage to the liver sinusoidal endothelial cells (SEC) [24, 139-141]. The result of cold storage ischemia injury is cellular death via a combination of apoptosis and necrosis [24, 142], which leads to sinusoidal narrowing, platelet adhesion and reduced microcirculatory flow on reperfusion [21]. The process of warm organ damage, occurring *in situ* in low flow states, is dominated by resident immune cell-derived cytotoxic molecule mediated hepatocellular injury, which leads to increased microcirculatory disturbances, cell dysfunction and inflammation [143-147]. The ischemic injury, a localized process of hepatic metabolic disturbances, is resulted by glycogen consumption, lack of oxygen supply and ATP depletion. In the reperfusion injury, an immune activation mediated amplification process, alarmins are produced during the initial cellular insult. The alarmins trigger liver nonparenchymal cells (NPC) to generate the pro-inflammatory milieu and can damage the liver tissue not only directly, but also indirectly by activating neutrophils and recruiting immune cells from the circulation [148]. These seemingly distinct processes share common mechanisms of sterile inflammatory immune cell activation, in that PRRs or specific alarmin receptors expressed by innate immune cells undergo activation following ligation of released endogenous danger signals [149, 150]. Liver contains abundant and heterogenous immune cell populations, including the highest content of tissue resident macrophages, that are Kupffer cells (KC), as well as DC, natural killer (NK) cells, natural killer T (NKT) cells, MC and T cells, that participate in maintaining homeostasis as well as responding to hepatic injury. These cells play key roles in the

initiation and progression of liver diseases [151]. Liver resident cells function together with infiltrating myeloid and lymphoid cells to respond to endogenous danger signal stimulation during IRI. The resulting inflammatory milieu further recruits and activates innate and adaptive immune cells to amplify such responses. The common and specific reactions of relevant hepatic immune cell subsets will be discussed herein.

1.4.1 Kupffer cells (KC)

Liver has an abundant macrophage population and is also patrolled by blood monocytes, which scan the liver vasculature and can infiltrate into the liver tissue [152]. Under steady state conditions, these myeloid monocytes can develop into liver DC [153] or monocyte-derived macrophages, but do not contribute to the pool of local resident macrophages in the liver, termed KC [154]. KC are derived from resident stem cells originating from the fetal yolk sack, which are independent of the myeloid monocytic compartment [154, 155]. KC constitute more than 50% of the tissue macrophages present in the body and are the most abundant immune cells in liver. They compose up 15% of all hepatic cells and 35% of the nonparenchymal liver cells [156].

An excessive inflammatory response initiated by KC is undoubtedly recognized as a critical mechanism in liver IRI. KC produce reactive free radicals (including ROS and RNS) and pro-inflammatory cytokines (such as TNF, IL-6, IL-1β and IL-18 etc.) [157]. When the portal vein is interrupted during IR, endogenous alarmins are generated during cellular stress or cellular injury [149]. The accumulated danger signals in the liver subsequently activate KC during reperfusion. During the early stages of IR insult in the liver, KC are activated by endogenous danger signals, such as HMGB-1 or histones, and are thought to be the primary cellular sources of ROS [158, 159]. TLRs including TLR4

and TLR9 on KC are activated by hepatocellular danger signals [159, 160], and the subsequent ROS released by KC into the extracellular matrix recruit $CD4^+$ T cells to the site of injury. These $CD4^+$ T cells express CD154, which can reciprocally activate KC in a CD40 dependent manner, leading to a further increase in ROS and cytokine production [161, 162]. The pivotal role of KC in ROS production during IR is demonstrated by the finding that KC inactivation attenuates IR-induced oxidative stress [163]. In addition, oxidative damage following IR tends to occur in hepatic regions rich in KC [164]. The activation of KC in the vasculature leads to their release of additional pro-inflammatory cytokines, such as TNF, IL-1β, CXCL10, CCL2, and CXCL8, all of which serve to attract neutrophils, monocytes, lymphocytes and other types of phagocytes to the liver after reperfusion [25, 165, 166]. Conversely, nitric oxide (NO) derived from KC may reduce IRI due to its vasodilatory effects [167, 168]. Moreover, KC protect against IRI by upregulating their expression of heme-oxygenase 1 and eliminating pro-inflammatory factor accumulation in the liver microenvironment [169]. In addition, KC produce IL-10 in response to endotoxin and IRI after liver transplantation *in vitro* and *in vivo*, with IL-10 acting as one of the key anti-inflammatory cytokines capable of alleviating liver IRI [170]. Altogether, these data suggest that the role of KC in hepatic IRI may be self-limiting. KC are responsible for the initiation of the pro-inflammatory response in IRI and may also function to terminate their own initiated reaction.

1.4.2 Dendritic cells (DC)

Liver DC play a central role in driving hepatic immune responses either towards tolerance or inflammation. Liver DC in mice can be divided into conventional DC (cDC) or plasmacytoid DC (pDC). cDC, characterized by $CD11c^{hi}MHCII^{hi}$, have a pivotal role in

recognizing tissue injury or infection and have a superior ability to process and present antigens to T cells compared to pDC [171]. cDC can be further divided into two populations of cDC on the basis of CD103 and CD11b expression in mice [171]. CD11b$^+$ cDC, or "myeloid" DC, exhibit high levels of MHC class II and appear to have a prominent role in the induction of CD4$^+$ T cell-mediated immunity [172]. CD103$^+$ cDC in the liver are considered largely analogous to lymphoid organ CD8$^+$ DC in their origin and function. They are specialized in their ability to cross-present exogenous antigens on MHC class I molecules to prime CD8$^+$ cytotoxic T cells [173]. Plasmacytoid DC are a specialized DC population, highly abundant in the liver with an important role in viral defence. Unlike cDC, pDC express low levels of MHC class II and co-stimulatory molecules. When activated, pDC produce large amounts of type I IFNs [174]. In mice, pDC can be distinguished by the expression of markers such as B220, CD317 (also known as BST2 or PDCA-1), which are not expressed by other DC subsets [175]. Although pDC can accumulate in the blood and lymphoid tissues, previous findings suggest that pDC function during liver viral infection is maintained by intrahepatic pDC rather than trafficking pDC [176, 177].

Hepatic cDC also express classical PRRs such as TLR4 and TLR9. Selective deficiency of TLR4 in DC of mice leads to increased liver IRI compared with wild type (WT) mice [178]. This observation suggests that TLR4 plays a primarily anti-inflammatory role in hepatic DC, compared to TLR4 expressed by extrahepatic myeloid cells and hepatocytes where it has a pro-inflammatory function. Consistent with this, targeted depletion of CD11c$^+$ DC in mice increases the severity of liver IRI [179]. Furthermore, hepatic DC reduce the inflammatory response to liver IRI in mice by producing anti-

inflammatory IL-10 via TLR9 signaling [179]. These results suggest that hepatic DC responding to danger signals released during IR and may protect the liver from progressive tissue injury.

The IFN pathway is critical for liver inflammatory injury following IR. Liver pDC are the principal cellular source of type I IFN in response to liver IRI [180]. Different IFN signaling pathways mediate distinct effects during liver IR. IRF-1 knockout (KO) mice display enhanced liver IRI [181, 182]. However, interferon regulatory factors (IRF)-2 heterozygote KO (*IRF-2*$^{+/-}$) donor grafts suffer more severe IRI than the WT control in mouse liver transplantation, suggesting that intrahepatic IRF-2 is protective and its overexpression represses the induction of IRF-1-dependent genes and decreases subsequent liver injury [183, 184].

Other mechanisms appear to contribute to the protective role of hepatic DC in liver IRI. For example, CD4^{+} T cell and DC interaction in the post-ischemic liver attenuates further CD4^{+} T cell recruitment and reduces IRI [185, 186]. Multiple mechanisms may contribute to the regulation of the inflammatory response in liver IR by liver DC.

1.4.3 Natural killer cells (NK)

NK cells are a major population of lymphocytes in the liver, comprising 30~50% of the hepatic lymphocyte population [187-189]. Human NK cells are defined phenotypically by the expression of the adhesion molecule CD56 and the absence of CD3. The density of CD56 expression, together with Fc receptor expression, defines two distinct subpopulations, CD56-high-expressing (CD56^{bright}) and CD56-low-expressing (CD56^{dim}) NK cells [188, 190]. In the blood, CD56^{dim} account for 90% of NK cells with preferential cytolytic activities, and the 10% CD56^{bright} NK cells primarily release immunomodulatory

cytokines. However, over 45% of the hepatic NK cells are $CD56^{bright}$ [191] and are functionally distinct from their peripheral blood counterparts. $CD56^{bright}$ NK cells contain significantly more preformed granzymes and express higher levels of TRAIL, perforin, and granzyme B [189], but have a poor cytolytic capacity [192]. NK cells have limited constitutive functions within the healthy liver, but are important during liver inflammation, especially in anti-viral defence [193]. TLR4 signaling on resident liver cells, such as KC, indirectly silences patrolling NK cells by MYD88-dependent IL-10 secretion, whereas TLR2 or TLR3 activation, together with inflammasome activation, induces IL-18 and IL-1β expression, leading to NK cell activation [194]. With respect to liver IRI, it has been shown that bone marrow-derived NK cells contribute to liver damage in a CD39/IFN-γ dependent manner [195], whereas the protective role of NK cells in mouse liver IRI is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent mechanism. Mice lacking $NKp46^{+}$ NK cells show worse hepatocellular damage, suggesting a protective role of hepatic NK cells in liver IRI [196]. In general, different subsets of NK cells are considered functionally distinct in this context.

1.4.4 Natural killer T cells (NKT)

NKT cells are a subset of T lymphocytes that are particularly enriched within the liver compared with other organs. These cells regulate immune responses in the context of autoimmunity, cancer, microbial infection and a variety of liver diseases [197-199]. In mice, NKT cells make up approximately 30% of intrahepatic lymphocytes compared to nearly 10% in humans [187, 200, 201]. In contrast to traditional $CD4^{+}$ T lymphocytes, NKT cells can be activated by antigen presenting cells (liver DC and presumably also KC) via glycolipid presenting receptors such as CD1d. This triggers both IL-4 and IFN-γ release

from NKT cells with a dependency on IL-12 for the release of IFN-γ [202, 203]. Interestingly, CD1d is expressed by most cells in the liver. NKT cells constitutively express mRNA encoding several pro-inflammatory cytokines (e.g. TNF, IFN-γ, IL-6 and IL-4) [204] and therefore secrete cytokines rapidly upon stimulation [205, 206].

The number of NKT cells decreases during various experimental models of liver dysfunction and disease, such as in leptin-deficient mice, bacterial liver injury, hepatotoxic liver injury, liver steatosis, and concanavalin A-induced liver injury [207-213]. However, following liver transplantation, hepatic IRI, or liver resection, the number of hepatic NKT cells increase [214-219]. While the proportion and number of NKT cells peaked in the liver at 10-20h after reperfusion, the magnitude of liver injury is decreased by 50% in NKT cell deficient mice as early as 6h post-reperfusion [216]. Interestingly, NKT cells produce IFN-γ as early as 2h following reperfusion and NKT depletion reduces liver IRI, while adoptive transfer of NKT cells into lymphocyte-deficient mice restores the IRI to the level of wild-type (WT) animals [215, 220, 221]. Additionally, pre-activation of NKT cells by intraperitoneal injection of the glycolipid antigen α-galactosylceramide (α-GalCer) 60 minutes prior to hepatic ischemia results in a significant reduction in serum hepatic enzyme levels [222]. These observations clearly suggest an anti-inflammatory role for hepatic NKT cells in IRI.

1.4.5 T cells

T cells are believed to be responsible for graft rejection through allogeneic T cell responses. However, increasing observations have also defined a role for T lymphocytes in acute and subacute phases of liver IRI (Table 1). Hepatic T cells show remarkable heterogeneity regarding their diverse immunological profiles, enabling them to perform

multiple pro-inflammatory and anti-inflammatory functions in liver disease. To gain a mechanistic insight into T-cell functions in IRI, genetically modified mice carrying different transgenes or gene KO relevant to T-cell functions have been utilized. By using a murine model of hepatic IRI, Zwacka and colleagues first observed that T lymphocytes are key enhancers of subacute neutrophil inflammatory responses in liver IRI [223]. Using a partial liver ischemia model, they compared the extent of reperfusion injury between immune competent BALB/c and athymic nu/nu mice. The authors noted a similar level of acute injury (3-6h post-reperfusion), however, the subacute phase (16-20h) was dramatically different. Serum alanine aminotransferase (ALT) and the degree of histologic necrosis was significantly reduced in the T-cell-deficient mice, which was associated with a 10-fold reduction in neutrophil infiltration [223]. Similarly, antibody-mediated depletion of $CD4^+$ T cells in wild-type mice resulted in a reduction of subacute injury and inflammation. Depletion of $CD8^+$ T cells had no effect on transaminase levels or tissue injury [223]. By kinetic analysis of T cell infiltration in the livers of BALB/c mice, a five-fold increase in the number of hepatic $CD4^+$ T cells was observed within the first hour of reperfusion, whereas there was no significant change in the number of $CD8^+$ T cells [223]. In 2002, a study found a dramatic reduction in serum ALT at just 4h reperfusion in athymic mice (nu/nu) [224]. In addition to resident T cells, peripheral T lymphocytes were recruited to the liver as early as 60min following IRI [225]. Contrary to previous observations, mice that lacked $CD4^+$ T cells demonstrated a more robust biochemical injury (detected by ALT levels) at 8h of reperfusion than wild type mice after 90min of initial ischemia. These findings were paradoxically associated with a reduction in neutrophil accumulation at the same time point [226]. Ultimately, it remains to be further defined exactly when and how

different subsets of T lymphocytes promote tissue injury or protect tissue injury in liver IRI, but it is clear that they have a broader impact than initially thought.

Table 1. $CD4^+$ T lymphocytes and liver ischemia and reperfusion (IRI).

Murine Models of IRI	Findings
Partial ischemia 30/60/90min and reperfusion from 0 to 36h	Similar IRI in acute phase (3-6h) between BALB/c and nu/nu mice. At 16-20h enzymes/necrosis reduced in nu/nu (10-fold reduction in neutrophil accumulation). $CD4^+$-depletion in BALB/c reduced IRI [223] .
Partial ischemia 90min and reperfusion at 4 and 20h	IRI significantly reduced in T-cell deficiency, disruption of CD154 signaling, or CD154 blockade [224].
Partial ischemia 90min and reperfusion at 1, 4, and 8h	$CD4^+$ T cells were recruited to the liver within 1h of reperfusion. $CD4^{-/-}$ had greater injury and less neutrophil accumulation [227].
Partial ischemia 90min and reperfusion at 30min and 140min	$CD4^+$-deficiency, CD40-CD40L and CD28-B7 disruption attenuates platelet adherence, reduces neutrophil transmigration, sinusoidal perfusion failure, and transaminase activities [228].
Partial ischemia 90min and reperfusion at 8 h.	$CD4^{-/-}$ and $CD4^+$-depletion protect from IRI. CD154 blockade protects from IRI. CD4 T cells function in IRI without de novo antigen-specific activation [229].
Partial ischemia 60min and reperfusion 0 to 24 h	Anti-CD25 antibody protects from IRI via reducing $CD4^+$ T cells (less expression of TNF, IFN-γ, IL-2, and IL-6) [230].

1.4.6 Mast cells (MC)

MC are a key component of innate immunity in part due to their strategic location at sites which interface with the external environment and association with blood vessels. Hepatic MC are mainly associated with the connective tissue that is found near hepatic arteries, veins, and bile ducts of the portal tracts [231-233]. In normal rodent and human livers, MC accumulate in low numbers along the portal tracts, indicating their presence is not solely based on liver injury [231-233]. However, infiltrating hepatic MC are found in large numbers near portal tracts and damaged bile ducts during different hepatic injuries, including hepatic fibrosis, cholangiopathies, liver cancer, alcoholic liver injury, fatty liver disease and allograft rejection [234-252].

MC have historically been mainly known for their roles in allergic disease, but are increasingly recognized as key regulatory cells involved in the inflammatory process [253-255]. MC recognize danger signals through the expression of multiple innate immune receptors and have the capacity to initiate inflammatory responses. MC can play both pro-inflammatory and anti-inflammatory roles, and an important aspect of their function is the recruitment of other immune cells such as neutrophils, eosinophils and T cells through cytokine and chemokine production [256-262]. MC are characterized by a complex response to stimuli, which can involve rapid degranulation and release of preformed inflammatory mediators within minutes [263]. This process is typically associated with de novo synthesis of lipid mediators and a slower, but sustained, secretion of a vast array of newly synthesized cytokines and chemokines over several hours [264, 265], although these processes can also occur independently. Two main classes of receptors are utilized in degranulation responses: 1) Fc receptors (FcRs) that allow MC to specifically respond to

antibody-targeted antigens and 2) G protein-coupled receptors (GPCR) that allow MC to degranulate in response to soluble mediators and other immune stimuli [266]. MC are tissue-based stationary effector cells that present first-line defense against various challenges as they are closely related to the inflammatory injury process [267]. In general, distinct MC activation events elicit distinct inflammatory mediator responses [268].

The specific role of MC during liver transplantation has been of increasing interest to researchers in recent years [269-272]. In a rat liver transplant model, MC were observed to co-localize with regulatory T cells (Treg), γδ T cells, and hepatic progenitors largely in tolerated livers compared to rejected livers [269]. *In vitro*, MC were able to increase the γδ T cell population following compound 48/80 stimulation associated with degranulation and increase hepatocyte proliferation, which suggested that MC-secreted factors can induce proliferation in various cell types [269]. This study highlighted the beneficial role of MC in the immunotolerance of transplanted livers. In contrast, in liver biopsy studies, an increase in the density of portal tract MC was observed in acutely rejected livers compared with controls and a further increase in MC was observed in chronically rejected compared with acutely rejected liver [270, 271]. In addition, preconditioning donor livers with cromolyn or compound 48/80 to stabilize or deplete MC reduced acute rejection and prolonged survival of recipients in a rat orthotopic liver transplantation model [272]. Therefore, the current understanding of the role of MC during liver tolerance/rejection is controversial and remains mostly unclear.

MC are function diversely and implicate in the pathogenesis of several types of IRI. In mouse models of myocardial infarction, the IR heart transiently produces cardioprotective polypeptide, but this is rapidly degraded by MC derived chymase, MC

protease 4. Mouse MC protease 4 deletion promotes pro-survival signaling in cardiomyocytes, which ameliorates cardiac dysfunction caused by ischemic injury [273]. Moreover, MC deficiency or pharmacologic stabilization in mice leads to a less severe injury during intestinal IR [10]. During renal IR, MC play a deleterious role in the acute inflammatory phase, promoting subsequent fibrosis development [274]. In previous studies, only rats within the rodent group were utilized as research subjects for hepatic IR-associated MC due to the presence of very few MC in mouse liver. These studies suggested an adverse role in liver IRI [275].

A recent study found that in a mouse model, the gastrointestinal MC were activated during liver IRI, which was proposed to exert a deleterious effect on livers subjected to IR, in light of the finding that MC deletion reduces the degree of liver injury [276]. This study is of particular interest because local liver IRI promoted MC degranulation in the stomach and intestine, which suggests there are danger signals released during liver IRI that might be potent MC activators. The activation of MC by alarmins released by liver IRI could further contribute damage to the liver [276]. The mechanisms underlying MC activation in hepatic IRI remain to be established but have been examined in the studies described later in this thesis.

1.5 Immune sensors for extracellular FM

The infiltration of neutrophils is commonly seen in liver IRI, which can aggravate liver IRI by secretion of pro-inflammatory mediators [277]. Previous studies suggested that the recruitment of neutrophils is regarded as a secondary response to mediators released by activated hepatic resident immune cells, such as KC [278-281]. It is now well accepted that mitochondrial alarmins are released from necrotic cells or damaged tissues and have numerous far-reaching effects on the acute inflammatory response. Numerous studies have found that mitochondrial alarmins are detected directly by neutrophils. Mitochondrial lysate (MTDs) containing NFPs from the crude sonicated mitochondrial preparations injected intravenously can cause neutrophil mediated attack on the lung [82, 282] in an FPR-dependent mechanisms [283]. Furthermore, intact extracellular mitochondria induce neutrophil ultrastructural changes and the release of neutrophil extracellular traps (NET) *in vitro* [135]. NETs, which contain released neutrophil mitochondria in the extracellular space, were also observed in liver IRI and further exacerbate liver injury [284]. Such extracellular mitochondrial components can cause inflammatory injury by mobilization and activation of neutrophils. However, it has not yet been determined whether the damaged liver during IR would release mitochondrial alarmins that direct neutrophil migration and instigate the oxidative burst.

Probably the best studied mitochondrial alarmin is mtDNA. mtDNA can be released into the circulation when there is cell damage or stress and is easily quantifiable in a simple inexpensive procedure. For example, Boudreau *et al* . quantified mtDNA in the extracellular milieu of platelet concentrates that induced adverse reactions and compared the levels within samples that were transfused without incidents [135]. Pollara *et al* .

examined the plasma of deceased organ donors for liver transplant and found elevated levels of mtDNA [134]. Interestingly, the authors confirmed that significantly higher levels of mtDNA are correlated with adverse reactions. Importantly, both Boudreau *et al* . and Pollara *et al* . found that intact extracellular FM, not organelle-free mtDNA, were the major source of mtDNA [134, 135]. If the intact extracellular FM are the major source of mitochondria alarmins in some pathological conditions, how are sensor cells recognizing the NFPs or mtDNA which are still cantained within the double mitochondrial membrane? In a study examining the release of extracellular mitochondria from platelets, it was observed that an enzyme, secretory phospholipase A2 (sPLA2) family member called IIA (sPLA2-IIA), could hydrolyze the naked mitochondrial membrane and thereby release mtDNA and other mitochondria alarmins, which are known triggers of immune responses and induce acute lung injury [135]. In contrast, in a recent study of *in vitro* co-culture of intact mitochondria with primary human aortic endothelial cells (HAEC), it was shown that the HAEC could takeup intact mitochondria via phagocytic mechanisms [285]. Inhibitors of phagocytosis including poly-I (scavenger receptor inhibitor) and cytochalasin E (inhibitor of actin polymerization) prevented mitochondrial uptake by endothelial cells (EC), which in turn inhibited EC activation.

As mentioned above, multiple cellular lineages such as neutrophils [120-122], eosinophils [123], MC [124], T cells [125], hepatocytes [84, 126], and platelets [127, 128] are reported at sites of intact mitochondria release, including the liver. Whether these cells could be activated by released mitochondria in an autocrine manner remains to be determined.

1.6 Hypotheses and objectives

Given that advances in the field of liver transplantation have led to it becoming the therapy of choice for those with end-stage liver disease or certain hepatic cancers not amenable to liver resection. The number of patients who are candidates for this life-saving therapy far outweighs the availability of donor organs. As wait lists have grown and more patients die while waiting for an organ, transplant surgeons have become more aggressive in using organs previously thought to be too high-risk for transplantation. Limiting liver IRI plays a dominant role in the procedure of liver transplantation, but also remains a limitation for the use of liver donor pools.

In the **first part** of my study, I sought to investigate the role of mitochondria alarmins in the pathogenesis of hepatic IRI. **Aim I** of this study was to determine **whether mitochondrial alarmins are released during hepatic IRI by quantification of mtDNA or assessment of extracellular mitochondria using flow cytometry or transmission electron microscopy**. We used *in vitro* models of cold and warm IR and confirmed the obtained findings with an *in vivo* model of hepatic IR. **Aim II was to investigate whether mitochondrial alarmins can induce hepatocyte damage, irrespective of IR**. This study could help us to understand the role for mitochondrial alarmins in the pathogenesis of hepatic IRI and demonstrates its potential as a biomarker of injury following IR.

Recent studies have revealed that extracellular mitochondria are critically involved in the initiation and progression of various diseases (not limited to liver IRI) and associated with immune dysregulation. MC are versatile immune sentinel cells known to respond to both external and internal stimuli as a first line of defense. However, the response of MC to extracellular mitochondria is unknown. More importantly, sPLA2-IIA has been

suggested to play a critical role in hydrolyzing naked mitochondrial membrane to release encapsulated mitochondrial alarmins [135]. Notably, sPLA2-IIA is stored in the secretory granules in MC and could be rapidly released to the extracellular milieu during MC degranulation [286]. Overall, these findings suggested that MC might play an important role in inflammatory responses initiated mitochondrial alarmins. **Therefore, in the second part of my study, I sought to dissect both human and mouse MC responses to extracellular mitochondria with a view to better understanding the role of MC in inflammatory responses to tissue damage involving the release of mitochondria**.

KC are liver tissue macrophages with the phagocytotic ability to remove endogenous danger signals from the circulation [287-289]. KC are the most abundant resident immune cells in liver with both inflammatory and anti-inflammatory immune effects, and are closely related to the pathogenesis of liver IRI, but their response to mitochondrial signals are poorly understood. Therefore, **in the third part of my PhD dissertation, the macrophages response to extracellular mitochondrial alarmins was assessed**. This characterization will help us better understand the mechanisms of liver IRI and other sterile inflammatory responses in macrophage rich tissues.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell lines and reagents

The McA-RH7777 rat hepatocyte, Hep G2 human hepatocyte, L929 mouse fibroblast, WEHI-3 myelomonocytic cell and RAW264.7 mouse macrophage cell lines were originally purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA).

N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF), Ethylene-bis (oxyethylenenitrilo) tetraacetic acid (EGTA), calcium ionophore (A23187), protease inhibitor cocktail and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclosporin H was obtained from LKT Laboratories (St. Paul, MN, USA). Boc-1, WKYMVm peptide, WRW4 were obtained from TOCRIS Bioscience Canada (Oakville, ON, Canada). The inhibitors FR180204(ERK 1/2 p38 inhibitors) and 420116 (JNK inhibitor) were purchased from EMD Millipore (St. Louis, MO, USA). KIN001-102 (Akt kinase inhibitor) and BMS-345541 [I kappa B kinase (IKK) inhibitor] were purchased from Sigma-Aldrich. Torin and rapamycin (mTOR inhibitors) were purchased from TargetMol USA.

2.2 Cells cultures

2.2.1 Cell line cultures and conditioned medium

McA-RH7777, Hep G2 and RAW264.7 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Gibco, Canada), 2mM L-glutamine (Gibco, Canada), 100U/ml penicillin and 100mg/ml streptomycin (Gibco, Canada) in a humidified 5% CO_2 atmosphere.

WEHI-3B cells constitutively produce high levels of IL-3 [290] which have been cultured in many laboratories to provide a relatively inexpensive source of IL-3 used for MC cultures mainly derived from bone marrow stem cells [291]. WEHI-3 cells were cultured at a concentration of $0.2x10^6$ cell/ml in Roswell Park Memorial Institute 1640 medium (RPMI 1640, GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 5% FBS, 1 mM MEM non-essential amino acids (Life technologies, Logan, UT, USA), 10 mM HEPES (HyClone, USA), 100 U/ml penicillin, 100 pg/ml streptomycin (HyClone, USA), and 50 pM 2- mercaptoethanol (2-ME, Sigma Aldrich). Two days after culture, cell culture supernatant was collected and passed through a 0.45 pm filter to remove any residual cells. To obtain 10 X concentrated WEHI-conditioned medium, the supernatant was transferred into dialysis tubing (VWR, Mississauga, ON, Canada) and then put into a plastic bag surrounded with aquicide II powder (Calbiochem-Novabiochem, La Jolla, CA), and set at 4 °C until about 1/10 of the original volume was left in the tubing. This process usually takes about 1 to 2 days. The concentrated WEHI-conditioned medium was stored at -20 °C for later use.

L929 cells are used to produce macrophage colony-stimulating factor (M-CSF) for bone marrow-derived macrophages. Briefly, 4.7×10^5 L929 cells were seeded in a 75-cm^2 flask containing 55 mL of DMEM medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% heat-inactivated FBS (Gibco), 100U/ml penicillin and 100mg/ml streptomycin (Gibco). Cells were grown in a humidified incubator with 5% CO2 at 37°C for 7 days and supernatant was collected and filtered through a 0.45-µm filter. 50mL aliquots were frozen at -20°C (labeled as L929-conditioned medium) for later use.

2.2.2 Human cord blood derived mast cells (CBMC)

Human umbilical cord blood was obtained following maternal written consent and the data analyzed anonymously in accordance with the procedures recommended by the Izaak Walton Killam Health Center Research Ethics Board Halifax, NS, Canada (Approved protocol number: IWK HE 1005110). Human CBMC were generated by long-term culture of cord blood-derived cells according to an adaptation of the methods described by Enoksson *et. al* [292]. Briefly, mononuclear cells obtained from umbilical cord blood were cultured at 1.0×10^6 cells/ml and passaged once per week for 4 weeks in StemSpan SFEM medium (Stem Cell Technologies, Vancouver, BC, Canada) containing 10 ng/ml hIL-3 (supplemented in the medium only during the first week of culture, eBiosciences, San Diego, CA), 10 ng/ml hIL-6 and 100 ng/ml hSCF (Peprotech, Rocky Hill, NJ) in 5% CO_2 at 37°C. On the 5th week of culture, the medium was replaced by RPMI 1640 (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% FBS (Sigma Aldrich), 2 mM L-glutamine (HyClone Labs, Rockford, IL), 100 U/ml penicillin G, 100 µg/ml streptomycin (HyClone), 5×10^{-5} M 2-ME Sigma Aldrich), 15 mM HEPES (HyClone), 0.1 mM non-essential amino acids (HyClone), 100 ng/ml hSCF and 10 ng/ml hIL-6. The purity of MC used in the experiments was >95% CD117$^+$ as assessed by flow cytometry using anti-CD117 (c-kit)-APC (clone 104D2, BioLegend, San Diego, CA) beginning of week 6. The purity of MC used in the experiments was ≥95%.

2.2.3 Mouse bone marrow-derived mast cells (BMMC)

BMMC were generated from C57BL/6 mice using a modification of the method described by Tertian *et. al* [293]. Briefly, bone marrow was flushed from the femur and tibia of mice and cultured in RPMI 1640 (GE Healthcare Life Sciences, Logan, UT, USA)

supplemented with 10% FBS (Sigma Aldrich), 100 U/ml penicillin G, 100 μg/ml streptomycin (HyClone), 5×10^{-5} M 2-ME (Sigma), 200 μM PGE_2 (Sigma Aldrich) and 20% WEHI 3B cell culture supernatant (ATCC) as a source of mouse IL-3. After 4 weeks, the purity of MC was assessed by flow cytometry using anti-CD117-PE (clone 2B8, BioLegend, San Diego, CA). BMMC were used when purity was >95%.

2.2.4 Mouse bone marrow-derived macrophages (BMDM)

BMDM were generated from C57BL/6 mice using a modification of the method described by Weischenfeldt and Porse [294]. Briefly, bone marrow was flushed from the femur and tibia of mice using 5 ml DMEM (GE Healthcare Life Sciences, Logan, UT, USA) with 10% FBS (Sigma Aldrich) in a 25 5/8 G needle. Cell suspension was then passed through a 100 μm strainer. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin (Gibco) and 20% L929 cell culture supernatant (ATCC) as a source of mouse M-CSF. After one weeks, the purity of macrophages was assessed by flow cytometry using anti-CD11b-APC (clone M1/70, eBioscience) and anti-F4/80-PE (clone BM8, eBioscience). BMDM were used when purity was >95%.

2.2.5 Mouse Kupffer cell

BMMC were generated from C57BL/6 mice using a modification of the method described by Liu et. al [295]. Mouse liver was perfused by using Liver Perfusion Medium (Life Technologies) Then the liver was dissociated in DMEM medium with Liberase TM Research Grade (Roche), low glucose, 1× Pen-Strep and 15 mM HEPES. Filter the cell suspension through a 100-μm cell strainer and collect the filtered cells in a 50-mL conical tube. Spin the cells at 50×g for 5 min at 4 °C to get rid of hepatocytes. Stain the non-

parenchymal cells with anti-F4/80-PE antibody, then positive select Kupffer cell cells by using Mouse anti-PE MicroBeads (Miltenyi Biotec). The function of Kupffer cells was assessed by LPS stimulation.

2.3 Animals

All animal experiments were pre-approved by the Dalhousie University Committee on Laboratory Animals. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals issued by the Canadian Council on Animal Care.

Male Lewis rats (250-300 g) were purchased from Charles River (Sherbrooke, QC, Canada. Rats were housed 2 per cage in a pathogen-free facility with a 12-h light-dark cycle. MC-deficient C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) mice were bred from C57BL/6, B6.Cg-$^{KitW\text{-}sh}$/HNihrJaeBsmGlliJ (Wsh) mice obtained from Jackson Laboratories (Bar Harbor, ME, USA). Age and sex matched C57BL/6 control mice were bred in house or, for some experiments, purchased from Charles River Laboratories. All mice used for experiments were 8-12 weeks of age and housed in specific pathogen-free conditions at the Carleton Animal Care Facility, Dalhousie University. Water and food were available ad libitum.

2.4 Ischemia reperfusion (IR) models

2.4.1 IR *in vitro* models

For exposure to IR, culture dishes were placed in a Modular Incubator Chamber (MIC-101, Billups-Rothenberg. Inc., Del Mar, CA), functioning as an airtight chamber according to the manufacturer's instructions. To simulate cold IR, hepatocytes were cultured in University of Wisconsin (UW) cold preservation solution at 4°C incubator for 6 h. Reperfusion was then simulated by changing the UW solution to warm complete

DMEM medium, removing the plates from the hypoxic chamber and placing them in a normoxic, humidified incubator for another 1 h. To mimic warm IR alone *in vitro*, cells were cultured in the DMEM in the hypoxic chamber at 37°C for 2 h, then reoxygenated by the same way as transplantation IR *in vitro*. Rat hepatocyte cell line (McA RH-7777) and human hepatocyte cell line (HepG2) were investigated in the *in vitro* models described above, respectively: (1) Control group (2) Ischemia (Isc) group (3) Ischemia reperfusion (IR) group.

2.4.2 Total hepatic warm IR

Total hepatic IR was performed as previously described with minor modifications [296]. In brief, rats underwent a laparotomy and a sterile atraumatic clip was placed on the portal triad for 60 min to induce total hepatic ischemia and mesenteric congestion. Reperfusion was initiated by removal of the clamp. Animals were randomly allocated to two groups (n=3 each): (1) Control group undergoing a sham operation involving laparotomy without clamping of the portal triad and (2) Experimental group undergoing *in vivo* IR as described. 100 µl blood was drawn from the right atrium of the heart catheter every 30 min through the inferior vena cava (IVC). The rectal temperature was maintained at 37 °C throughout surgery by a warming pad. Sham controls underwent anesthesia, laparotomy, and exposure of the portal triad without vascular occlusion. At the end of the predetermined period following reperfusion, the animals were euthanized by cervical dislocation for tissue and plasma collection.

2.4.3 Segmental hepatic ischemia

A model of segmental (70%) warm hepatic ischemia was used, which was performed as previously described with minor modifications [160]. Briefly, under isoflurane anesthesia through inhalation, an upper midline abdominal incision was made. The vasculature supplying the left and median lobes (ischemic lobes) of the liver was occluded with a microvascular clamp (Fine Science Tools) for one hour. Evidence of ischemia during the clamping period was confirmed by blanching of the ischemic lobes. Reperfusion was initiated by removal of the clamp, and gross evidence of reperfusion was confirmed by color change of the ischemic lobes. Sham mice underwent the same procedure without the application of a vascular clamp. All mice received 40mg/kg of sterile sodium pentobarbital subcutaneously 30min prior to abdominal closure. The temperature during the procedure was maintained with the use of a warming pad and heating lamp. At the end of the observation period, mice were euthanized by carbon dioxide inhalation.

2.5 Cell viability

2.5.1 Apoptosis assay

Rat and human hepatocytes, McA-RH7777 and HepG2, were labeled with fluorescein isothiocyanate (FITC) labeled Annexin V and propidium iodide (PI) using an Annexin V-FITC apoptosis detection kit (eBioscience) according to the manufacturer's instructions. Briefly, after 24h exposure to different concentrations of MTDs (0, 40, 100, 200, and 400 μg/ml), or after different treatments (Ischemia, IR, co-culture with mtDNA or MTDs) the cells were washed with cold PBS and then resuspended in 1X binding buffer. Aliquots of $2x10^5$ cells were mixed with 5 μl Annexin V-FITC (0.25μg/ml) and 10 μl

propidium iodide (20μg/ml) for 10 min at room temperature in the dark. Fluorescence was detected within 1h using flow cytometry.

2.5.2 Immunohistochemistry for caspase-3

Apoptotic hepatocytes were identified using cleaved-caspase-3 / pro-caspase-3 immunohistochemistry. For caspase-3 immunostaining, 5μm-sections were dewaxed and hydrated through graded ethanol solutions, cooked in 10 mM citrate buffer at pH 6.0 in a pressure cooker/antigen retriever at 125 °C for 30 min (2100-Retriever, Electron Microscopy Sciences, Hatfield, PA, USA), then transferred into water to cool for 10 min. After 5 min of treatment in 3 % H_2O_2, the slides were blocked with 10% normal goat serum (NGS) for 1 h. After blocking with 10% NGS, the slides were dried, and covered with the primary antibody (caspase-3 rabbit monoclonal antibody 1: 800 and pro-caspase-3 rabbit polyclonal antibodies 1:2000, Cell Signaling) overnight at 4 °C. The slides were then washed three times and incubated with the secondary antibody (biotinylated goat anti-rabbit) for 1 hr. Following this, the slides were washed and dried, and then covered with ABC solution (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1h followed by the addition of DAB solution (Vector Laboratories) for 2 min. Later, DAB solution was washed and counterstained with Mayer's Haematoxylin for 2 min followed by rinsing with tap water 2-3 times. The slides were then immersed in Scott's solution for 2 min and dehydrated and mounted with a coverslip.

2.5.3 MTT Assay for Cell Viability

The colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to determine cell viability. This system measures the reduction of the tetrazolium component into an insoluble, coloured formazan product by

the mitochondria of viable cells. The absorbance is measured spectrophotometrically, and the amount of colour produced is directly proportional to the number of viable cells. Supernatants were removed from cells that had been plated in a 96-well flat bottom tissue culture dish (Corning Incorporated), cells were incubated with 0.5 mg/ml MTT for 2 hrs and the purple formazan products were dissolved in 100 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich). The plates were read on a plate reader (BioTek Synergy HT) at 570 nm with a reference reading at 630 nm. The cells were treated with different extracellular mitochondrial components for 24 hours or DMSO vehicle. Percent viability was calculated as (mean absorbance treatment) / (mean absorbance control) × 100%.

2.6 Liver damage evaluation

2.6.1 Pathological evaluation

Liver samples were fixed with formalin and embedded with paraffin. Sections (5µm) were stained with haematoxylin-eosin (H&E). Pathological findings were assessed blinded to the group allocations. For each method used for detection of cellular damages, the number of injured cells was calculated using high power microscopy in 50 ± 5 fields (original magnification 10x) by morphological criteria (cell shrinkage, chromatin condensation, margination, apoptotic bodies, cell swelling, cell rupture).

2.6.2 Liver enzyme levels

Serum (0.5 ml) was collected at 1h post-reperfusion and kept on ice until processed. Levels of aspartate transaminase (AST) and ALT were determined in serum, in duplicate, using commercial kits (Sigma), according to the manufacturer's protocols.

2.7 Mitochondrial damage and homeostasis evaluation

2.7.1 Mitochondrial ROS detection assay

Cells were cultured in ischemia or ischemia and reperfusion conditions for different time points together with normal condition cultured cells as control. At the last 30 min of culture, cells were incubated with 1 μM MitoSOX (Thermo Fisher Scientific) at room temperature for 30 min, and then cells washed, and mitochondrial ROS levels measured by a flow cytometer.

2.7.2 Mitochondrial membrane potential assay

Cells were cultured in ischemia or ischemia and reperfusion conditions for different time points together with normal condition cultured cells as control. At the last 30 min of culture, cells were incubated with 100 nM Tetramethylrhodamine, methyl ester (TMRM, Thermo Fisher Scientific) at 37°C for 30 min. Cells were then washed, and mitochondrial membrane potential were measured by flow cytometer.

2.7.3 Mitochondrial damage assay by MitoTrackers

100nM MitoTacker Green and 100 nM MitoTracker Deep Red (Thermo Fisher Scientific) were used to stain cells that underwent different conditions at room temperature for 30 min. The mitochondrial damage was measured by a flow cytometer at excitation at 490nm and 644 nm, and emission at 516nm and 665nm, respectively.

2.7.4 Transmission Electron Microscopy (TEM)

Liver segments were fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide. After washing, the segments were incubated in 0.25-0.5% uranyl acetate, rinsed and dehydrated through acetone. Samples were further processed by technicians at the Dalhousie Faculty of Medicine Electron

Microscopy Facility. Segments were embedded in epon-araldite resin and sectioned using an ultramicrotome (Reichert Jung Ultracut E) with a diamond knife. Sections were stained with lead citrate and uranyl acetate before examination on a JOEL 1230 transmission electron microscope at an accelerating voltage of 80kV. Digital images were captured using a CCD digital camera.

2.8 Real-time PCR

The primers for the real-time PCR analysis of mtDNA were: Primers for human cytochrome B (forward 5'-ATGACCCCAATACGCAAAAT-3' and reverse 5'-CGAAGTTTCATCATGCGGAG-3'), human cytochrome C oxidase subunit III (forward 5'-ATGACCCACCAATCACATGC-3'and reverse5'-ATCACATGGCTAGGCCGGAG-3'), human NADH dehydrogenase-ubiquinone oxidoreductase chain 1 (forward 5'-ATACCCATGGCCAACCTCCT-3' and reverse 5'-GGGCCTTTGCGTAGTTGTAT-3'), human β-actin (forward 5'-AGCGGGAAATCGTGCGTG-3' and reverse 5'-CAGGGTACATGGTGGTGCC-3'), rat cytochrome B (forward 5'-TCCACTTCATCCTCCCATTC-3' and reverse 5'-CTGCGTCGGAGTTTAA TCCT-39), rat cytochrome C oxidase subunit III (forward 5'-ACATACCA AGGCCACCAAC-3' and reverse 5'-CAGAAAAATCCGGCAAAGAA-3'), rat NADH dehydrogenase-ubiquinone oxidoreductase chain 1 (forward 5'-CAATACCCCACCCCCTTATC-3' and reverse 5'-GAGGCTCATCCCGATCATAG-3') and rat GAPDH (forward 5'-GAAATCCCCTGGAGCTCTGT-3' and reverse 5'-CTGGCACCAGATGAAATGTG-3') were synthesized by Integrated DNA Technologies (IDT, Coralville, US). The following primers were used to determine mRNA expression levels: TLR2, 5'-GGGATACAGGCCGTCAAGAC-3' (forward) and 5'-

CAGGAGCAGATGAAATGGTTGT-3' (reverse); TLR4, 5'-CGCTCTGGCATCATCTTCAT-3' (forward) and 5'-CTCCTCAGGTCAAAGTTGTTGC-3' (reverse); TLR9, 5'-CCTGGCACACAATGACATTCA-3' (forward) and 5'-TAAAGGTCCTCCTCGTCCCA-3' (reverse); MyD88, 5'-GAGATCCGCGAGTTTGAGAC-3' (forward) and 5'-TTGTCTGTGGGACACTGCTC-3' (reverse); NF-κB, 5'-GAGGACTTGCTGAGGTTGG-3' (forward) and 5'-TGGGGTGGTTGATAAGGAGTG-3' (reverse); and glyceraldehyde phosphate dehydrogenase (GAPDH), 5'-GGCACAGTCAAGGCTGAGAATG-3' (forward) and 5'-ATGGTGGTGAAGACGCCAGTA-3' (reverse). Primer sequences have no significant homology with DNA found in any bacterial species published on BLAST. Samples that produced no PCR products after 40 cycles were considered 'undetectable' and the Ct number set to 40 for statistical purpose. All reactions were performed in a total volume of 20 µl containing 50 ng DNA and 1x SYBR Green. All reactions were done in duplicates and relative gene expression values determined using the 2^{-ddCt} method with Bio-rad iCycler iQ™ 5. For mtDNA quantification, total DNA were isolated and mtDNA copy numbers were normalized to the nuclear genome DNA telomerase reverse transcriptase gene or GAPDH gene.

2.9 Mitochondria isolation and preparation of MTDs and mtDNA

Mitochondria isolation kits for tissue and for cultured cells (Thermo Scientific™) were used to isolate mitochondria from mouse liver and from human hepatocytes ($HepG_2$) (ATCC), respectively, according to manufacturers' instructions. Isolated mitochondrial pellets were suspended in HBSS. Halt™ Protease Inhibitor Cocktail (1:100) (Thermo

Scientific) was added to the suspension. Suspensions of MTDs and mtDNA were prepared according to an adaptation of the methods described by Zhang *et. al* [82]. Briefly, mitochondria pellets were frozen and thawed ten times in liquid nitrogen, and the disrupted mitochondrial suspensions were then centrifuged at 12,000g for 10 min at 4°C followed by 100,000g at 4°C for 30 min. Residual supernatants were used as MTDs for experiments. Protein concentrations of FM and MTDs in solution were determined by Bradford Protein Assay (Bio-Rad). mtDNA was extracted from the isolated mitochondria using DNeasy Blood & Tissue kit (Qiagen, Mississauga, ON) and concentrations of mtDNA determined using a nanodrop spectrophotometer.

2.10 Peritoneal cavity injections, cell harvesting, and flow cytometry

C57BL/6 mice were injected with 100 µL of either saline (diluent) alone, containing FM suspension (5mg/kg), 1 µg of the TLR2 agonist, FSL-1 (EMC Microcollections, Tübingen, Germany) and/or Cyclosporin H (CsH, 2mg/kg, LKT Laboratories). After 16 hours, mice were euthanized and the peritoneal cells were harvested by i.p. injection and recovery of 4 ml PBS, 0.5% bovine serum albumin, 5mmol/L EDTA. Mouse peritoneal cells (PCC) were counted. Single-cell suspensions were stained with fixable viability dye eFluor506 (eBioscience), anti-CD19-BV510 (clone 6D5, BioLegend), anti-NK-1.1-PerCP-Cy5.5 (clone PK136, eBioscience), anti-CD11b-PE-eFL610 (clone M1/70, eBioscience), CD117 (c-kit)-PE (clone 2B8, BioLegend), anti-CD4-APC (clone GK1.5, eBioscience), anti-FcεRIa-APC eFL780 (clone 1-Mar, eBioscience), anti-CD8a-BV650 (clone 53-6.7, BD Bioscience), anti-CD3e-BV711 (clone 145-2C11, BD Bioscience), anti-Siglec-F-PE-CF594 (clone E50-2440, BD Bioscience), anti-CD11b-PE (clone M1/70, eBioscience), anti-F4/80-PE-Cy7 (clone BM8, eBioscience), anti-CD11c-APC (clone

N418, eBioscience), anti-MHCII-AF700 (clone M5/114.15.2, BioLegend), anti-Ly6C-APC-eF780 (clone HK1.4, eBioscience), anti-CD103-BV510 (clone M290, BD Bioscience), anti-Ly6G-BV605 (clone 1A8, BD Bioscience), anti-CD24-BV711 (clone M1/69, BD Bioscience), anti-B200-BV786 (clone RA3-6B2, BD Bioscience), and anti-CD69-FITC (clone H1.2F3, BioLegend). Stained cells were acquired for analysis using a BD LSRFortessa, and results were analyzed using FlowJo software.

2.11 MC activation *in vitro*

2.11.1 MC degranulation

CBMC (0.25×10^6/ml) or BMMC (2×10^6/ml) in modified HEPES-Tyrode's buffer were treated for 20 min with increasing doses of FM, other mitochondrial alarmins or calcium ionophore A23187 (5 µM), as a positive control. The level of degranulation was assessed via histamine release according to the method of Price [297]. Values are expressed as % degranulation, and were calculated using the following method: $(OD_{sup} - OD_{HTB}) \div [(OD_{sup} - OD_{HTB}) + (OD_{pellet} - OD_{HTB})] \times 100\%$. B-hexosaminidase was not used as a readout in these assays because of false positive signals derived from mitochondrial preparations.

2.11.2 MC long term secretion experiments

Prior to experiments, CBMC and BMMC were cultured overnight without hIL-6 or PGE2, respectively. Prior to activation, the CBMC or BMMC were washed twice and resuspended in the experimental medium. Either CBMC or BMMC at $1X10^6$ cell/ml were activated with calcium ionophore, A23187 at 0.1 µM as positive control at 37 °C for either 6 hours or up to 24 hours and were then centrifuged at 300 g for 10 min. In some of CsH or FPRs inhibitors' experiments, CBMC or BMMC were pre-treated with those drugs 20

min before incubation with Extracellular mitochondria components. Supernatants were harvested for cytokine detection by ELISA or Luminex array. In some experiments, cell pellets were harvested for RNA isolation and qPCR analysis. Both supernatant and pellet samples were stored at -20 °C for later analysis.

2.12 Macrophage polarization

BMDM were used for macrophage polarization using a modification of the method described by Ying *et al* [298]. On day 7, change to fresh stimulation medium: for M1 activation, use IMDM containing 10% FBS and 100 ng/ml LPS or 50 ng/ml IFNγ; for M2 activation, use IMDM containing 10% FBS with 10 ng/ml IL-4 and/or 10 ng/ml IL-13. Use antibodies to detect expression of cell surface antigens, including CD11b, F4/80, CD11c, CD206, CD69, CD80 or CD86 at various time points using standard flow cytometry staining procedures.

2.13 Immunoassays

Magnetic Luminex Screening Assay kits were purchased for human and mouse from R&D Systems and run according to manufacturer's instructions using provided reagents, which measured CXCL1, IL-1β, IL-1RA, IL-6, CCL2, MMP9, TNF, IFNγ, IL-10, IL-5, CXCL8, CCL4, CCL5, VEGF-A, CCL11, IL-12 p70, IL-18, CCL3, GM-CSF, IL-13, and CXCL10 for cell-free CBMC supernatants; and GM-CSF, TNF, IL-1β, IL-4, IL-6, IFNγ, CCL3, MMP9, CXCL1, CCL2, VEGF, IL-5, IL-13, CCL5 and CCL4 for murine samples. Samples were read using a BioPlex 200 system (BioRad) and analyzed with BioPlex Manager 6.0 software (BioRad). For statistical analysis, samples at or below the limit of detection were assigned the limit of detection value for the assay. Human CXCL8 and human CCL3 ELISA kits were used to measure CXCL8, CCL3 (R&D

Systems), a mouse IL-6 ELISA kit was used for test murine IL-6 production (BioLegend). Serum tumor necrosis factor (TNF), interleukin (IL)-6, and IL-10 levels in rats were detected by an enzyme-linked immunosorbent assay (ELISA) kit (R&D System).

2.14 Analysis of publicly available datasets

The National Center for Biotechnology Information (NCBI)'s Genome Expression Omnibus (GEO) database for liver transplant relevant studies published before December 31st, 2018 was searched. Five criteria were used to manually screen the datasets identified in GEO-NCBI, including: (1) original papers containing independent data which have been published in a peer-reviewed journal, (2) liver transplantation, (3) sample size is above 15, (4) the Affymetrix Human Genome U133 Plus 2.0 Array was used, which includes all genes of interest, (5) samples are from human patients. After applying the five filters, a total of 2 datasets were identified and downloaded. Only one of the databases focusing on the operational tolerance in liver transplantation was used in this study.

2.15 Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad, La Jolla, CA, USA). For normally distributed data, the two-tailed unpaired Student t test was used to determine the significance of the differences between two groups. For comparison of multiple groups, analysis of variance (ANOVA) was performed followed by post-hoc multiple comparisons of means. For data which did not pass a normality test, nonparametric Mann Whitney test was used to determine the significance of the differences between two groups. Nonparametric Kruskal-Wallis test was performed for comparisons of multiple groups, followed by Dunn's post-hoc multiple comparison test. All data are shown as the mean ± standard error of the mean (SEM) or standard deviation

(SD). P values <0.05 were considered statistically significant. The following symbols were used to denote statistical significance: * P < 0.05, ** P < 0.01, *** P < 0.001, ****, P< 0.0001.

CHAPTER 3 RESULTS

3.1 Liver IR disturbs mitochondrial homeostasis and induces mitochondria release to extracellular milieu

Parts of this chapter were included in the article "Mitochondrial damage-associated molecular patterns are released during hepatic IR and induce inflammatory responses" PLoS One. 2015 Oct 9; 10(10):e0140105. doi: 10.1371/journal.pone.0140105 [299].

3.1.1 IR *in vitro* disturbs mitochondrial homeostasis

The mechanisms of IRI are diverse and complex, but are typically found to converge upon increased mROS emission, mitochondrial permeability transition pore opening and initiation of cell death [300]. Emerging evidence suggests that mitochondrial damage characterized by mROS over-generation and mitochondrial membrane potential ($\Delta\Psi m$) loss is positively correlated with graft tissue impairment after transplant surgery [301, 302]. To examine whether the IR process could affect mitochondrial homeostasis, A modular incubator chamber flushed with nitrogen to mimic ischemia was used to stress cells in hypoxic conditions for several time periods (3h or 24), followed by transfer to a normoxic humidified incubator for a further time period (1h and 24h) to mimic reperfusion *in vitro*. Mitochondrial ROS (mROS) production was confirmed by MitoSOX staining and mitochondrial membrane potential changes examined by TMRM staining to evaluate mitochondrial homeostasis. Notably, both time points (3h and 24h) of ischemia and with 1h reperfusion were able to significantly enhance mROS production (Figure 5A) and induce $\Delta\Psi m$ loss (Figure 5B) compared to control.

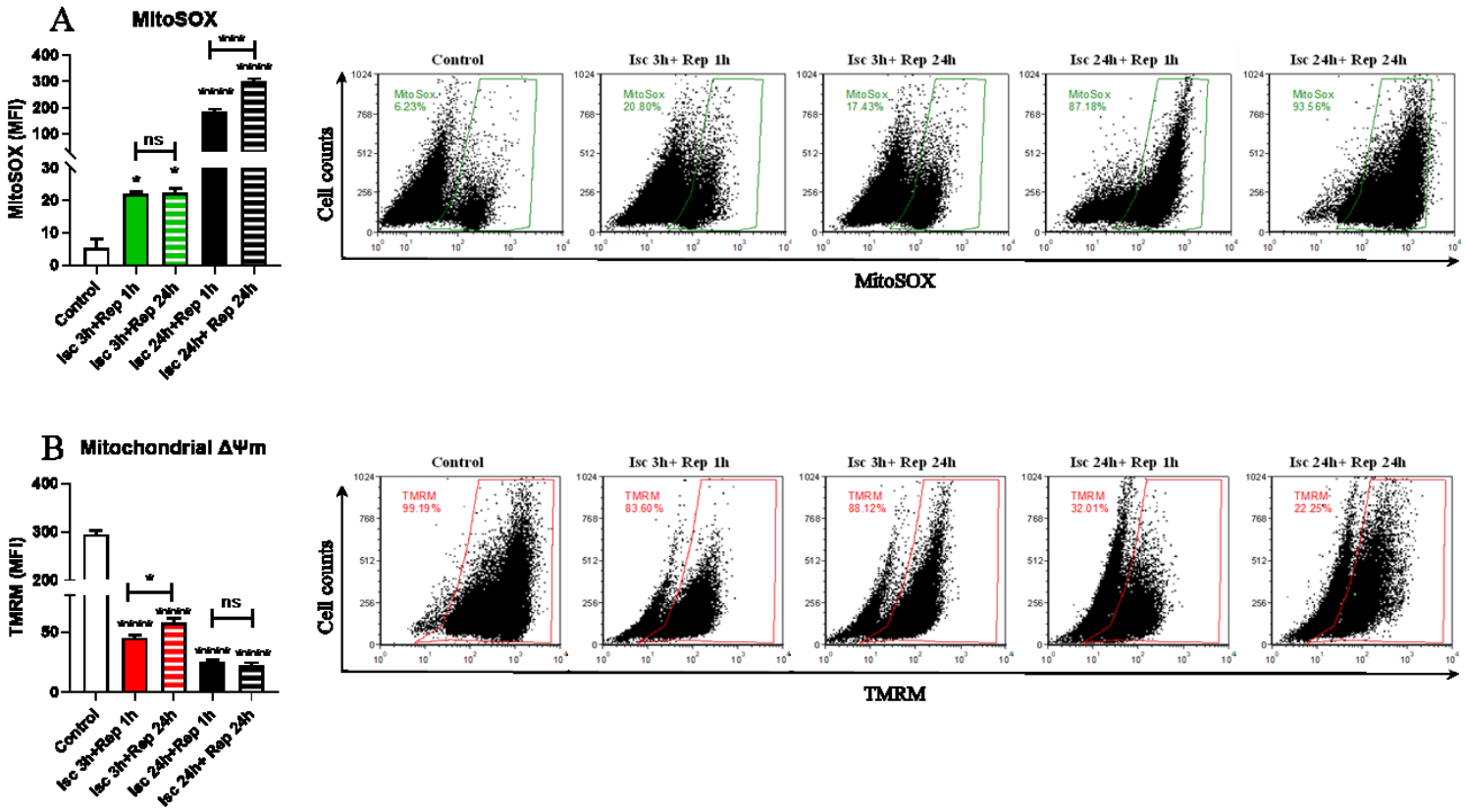
Next, to investigate whether the mitochondrial damage was reversible during a longer-term reperfusion, mitochondrial homeostasis was compared following a short term

(1h) and long term (24h) reperfusion after 3h and 24h ischemia stress conditions, respectively. For cells that underwent 3h ischemia, no matter whether this was followed with a 1h or 24h reperfusion, cells produced similar levels of mROS (Figure 5A, green columns) and interestingly the longer-term reperfusion (Isc 3h+ Rep 24h) helped cells recover ΔΨm loss compared to Isc 3h + Rep 1h group (Figure 5B, red columns). However, for cells undergoing 24h ischemia which had a more severe mitochondrial damage, a longer-term reperfusion (Rep 24h) further significantly enhanced mROS production and maintained a large ΔΨm loss compared to a short term of reperfusion (Rep 1h) (Figure 5A/B, black columns). Representative staining dot plots with percentage changes are shown in figure 5. Together, these data suggested that IRI *in vitro* significantly induced mROS production and mitochondrial ΔΨm loss, and severe IRI could dramatical alter mitochondrial homeostasis and worsen mitochondrial damage.

Figure 5. IR enhanced mROS production and mitochondrial membrane potential loss *in vitro*.

Mouse BMDM were stressed to 3h or 24h ischemia, then followed with re-oxygenation for 1h or 24h. Mitochondrial ROS production and mitochondrial membrane potential ($\Delta\Psi m$) state were determined by MitoSOX and tetramethylrhodamine, methyl ester (TMRM) staining, respectively, and then analysed by flow cytometry. (A) Mean fluorescence intensity (MFI) of MitoSOX staining of control and ischemia (Isc)/reperfusion (Rep) groups and representative dot plots stained with MitoSOX. (B) MFI of TMRM staining of control and Isc/Rep groups and representative dot plots stained with TMRM. All values are means ± SEM of 3 independent experiments. *, $P< 0.05$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 5


A
MitoSOX
MitoSOX (MFI)
ns
Control
Isc 3h+Rep 1h
Isc 3h+Rep 24h
Isc 24h+Rep 1h
Isc 24h+ Rep 24h
Cell counts
MitoSOX
MitoSox 6.23%
MitoSox 20.80%
MitoSox 17.43%
MitoSox 87.18%
MitoSox 93.56%
B
Mitochondrial ΔΨm
TMRM (MFI)
ns
TMRM
TMRM 99.19%
TMRM 83.60%
TMRM 88.12%
TMRM 32.01%
TMRM 22.25%

3.1.2 IR *in vitro* elevates mtDNA in both rodent and human hepatocyte cell lines

Previous studies have used mitochondrial DNA (mtDNA) as markers to assess mitochondrial components alteration and mitochondrial damage in several clinical conditions [69, 82]. To determine whether aberrant mitochondrial components play a role in hepatic organ damage after cold IR or warm IR, a similar *in vitro* IR model, as that described above, was used to stress both rodent and human hepatocyte cell lines at 4 °C or at 37 °C followed by transferring to a normoxic, humidified incubator at 37 °C.

First, rodent and human hepatocytes underwent cold ischemia for six hours in UW solution at 4 °C, after which they were transferred to a normoxic, humidified incubator for another 1h to simulate transplantation-related IR. Three independent experiments were performed for both cell lines (McA RH7777 and HepG2). Cells that experienced cold IR *in vitro* showed significantly higher mtDNA levels when compared to normoxic controls (Figure 6 A and B). In a second set of experiments, cell lines were exposed to warm ischemia alone for one hour and then reperfusion for another hour. Both rodent and human hepatocytes showed similar trends: there were significantly increased levels of mtDNA following ischemia, which was maintained after reperfusion (Figure 7 A and B). These results indicate that both cold and warm IR *in vitro* was associated with an elevation of mitochondrial components.

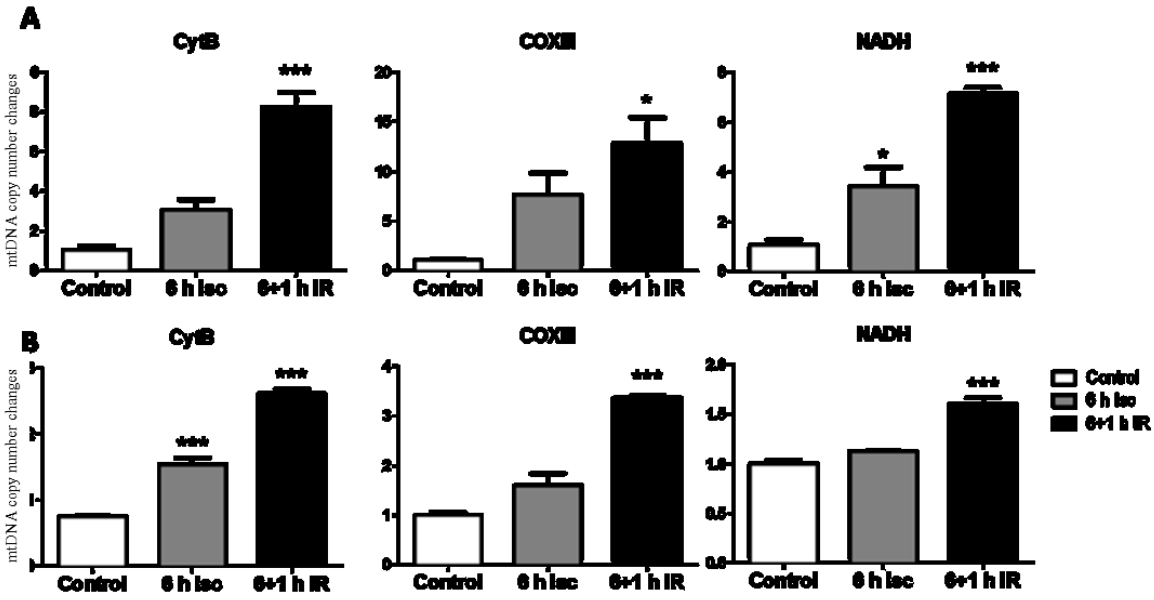


Figure 6. Transplantation-associated ischemia and warm reperfusion elevates mitochondrial DNA levels in both rodent and human hepatocyte cell lines *in vitro*.

A, six hours of transplantation-associated ischemia and one hour of reperfusion significantly increased the mtDNA levels in the rodent hepatocyte cell line, McA RH7777; B, six hours transplantation-associated ischemia and one-hour reperfusion significantly increased the mtDNA levels in the human hepatocyte cell line, HepG2.

Control: cells were cultured in a conventional cell culture incubator for seven hours; 6h Isc: six hours incubation in cold and ischemic conditions; 6+1h IR: six hours incubation in cold and ischemic conditions followed by one hour of warm reperfusion; CytB, mitochondrial cytochrome B; COXIII, mitochondrially encoded cytochrome c oxidase III; NADH, NADH dehydrogenase-ubiquinone oxidoreductase chain 1. All values are means ± SEM of 3 independent experiments. *, P<0.01, **, P<0.001, ***, P<0.0001.

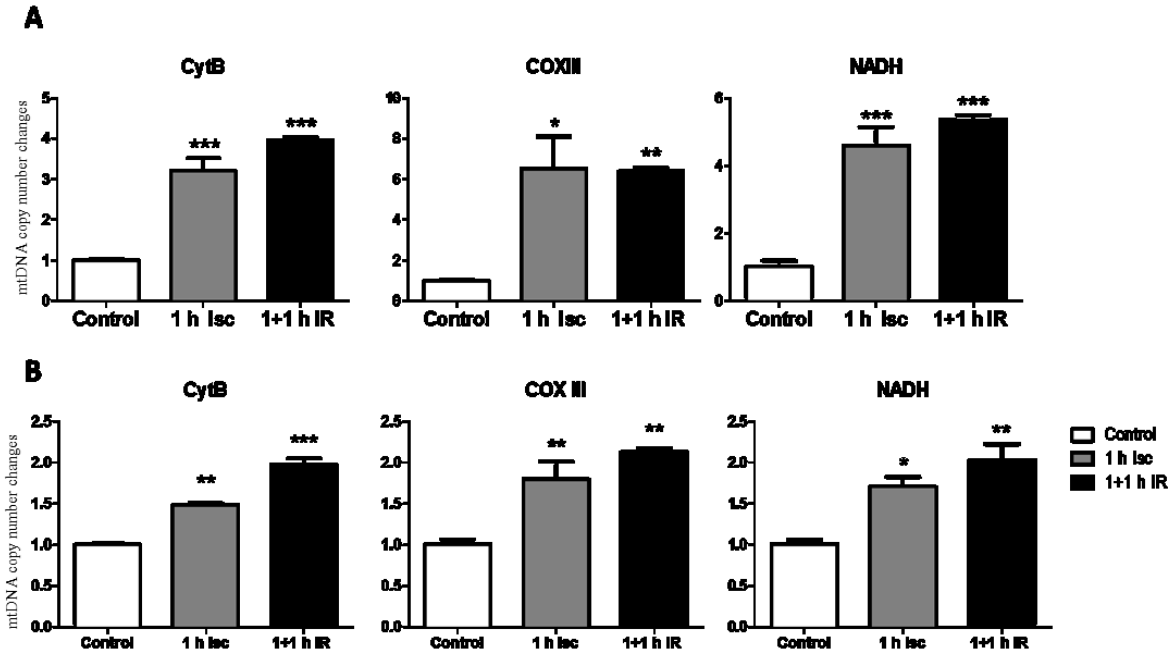


Figure 7. Warm ischemia and warm reperfusion *in vitro* elevate mitochondrial DNA levels in both rodent and human hepatocyte cell lines.

A, one-hour of warm ischemia and two hours of warm IR significantly increased the mtDNA levels in the rodent hepatocyte cell line, McA RH7777;

B, one-hour warm ischemia and two hours warm IR significantly increased the mtDNA levels in the human hepatocyte cell line, HepG2.

Control, cells were cultured in normal cell culture incubator for two hours; 1h Isc, one-hour warm ischemia incubation; 1+1 IR, one-hour warm ischemia incubation followed by one-hour warm reperfusion treatment, CytB, mitochondrial cytochrome B; COXIII, mitochondrially encoded cytochrome c oxidase III; NADH, NADH dehydrogenase-ubiquinone oxidoreductase chain 1. All values are means ± SEM of 3 independent experiments. *, P<0.01, **, P<0.001, ***, P<0.0001.

3.1.3 Mitochondrial components induce hepatocytes death and evoke inflammatory responses *in vitro*

To further explore the role of aberrant mitochondrial components in IR, MTDs were extracted from hepatocyte cell lines, and co-cultured with each hepatocyte cell line in normal, normoxic and normothermic environments. To determine if mitochondrial components would further induce cell death, the Annexin V-FITC apoptosis detection kit (eBioscience) was used to detect apoptosis or necrotic cell death following exposure of normal hepatocytes to MTDs and cells undergoing IR treatments with controls. Ischemia alone and IR treatments *in vitro* can trigger cell death to different extents (Figure 8 A-C). MTDs can induce a similar pattern of cell death as IR treatment *in vitro*, as there was a subpopulation of PI positive cells in both of the treatments (Figure 8C and D). These results suggest that MTDs might exacerbate organ injury after IR. I subsequently incubated hepatocytes with different concentrations of MTDs and observed that the MTDs trigger cell death in a dose dependent manner in both cell lines (Figure 9).

Figure 8. MTDs induce a similar pattern of cell death compared to cells undergoing transplantation-associated ischemia and warm reperfusion *in vitro.* Apoptosis assay *in vitro* in HepG2 hepatocytes undergoing IR treatment and MTD incubation.

A, Control: HepG2 hepatocytes were cultured in normal environment;

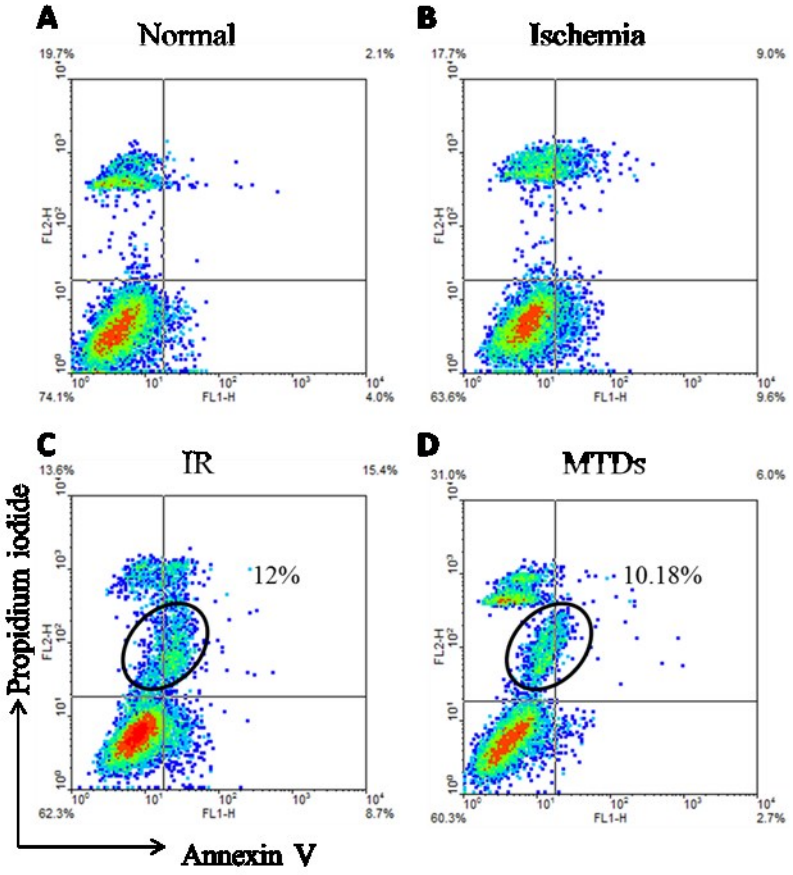
B, Ischemia group: HepG2 hepatocytes that were maintained in a hypoxic environment for six hours *in vitro* demonstrated increased apoptosis when compared to Control cells;

C, IR group: HepG2 hepatocytes that were incubated in a hypoxic environment for six hours and subsequently transferred to a normoxic incubator for 24 hours demonstrated an increased percentage of both apoptosis and necrosis. In addition, these conditions induced a sub-population of PI-positive cells (gated in circle);

D, MTDs incubation group: HepG2 hepatocytes that were co-cultured with 400μg/ml MTDs in a normal environment for 24 hours demonstrated an increased percentage of apoptosis and necrosis with an increased sub-population of PI-positive cells (gated in circle), similar to the IR group (C).

The figures shown are representative of three experiments with similar results. The PI single positive population is the basal level cell death.

Figure 8


A
Normal
19.7%
2.1%
74.1%
4.0%
B
Ischemia
17.7%
9.0%
63.6%
9.6%
C
IR
13.6%
15.4%
12%
62.3%
8.7%
D
MTDs
31.0%
6.0%
10.18%
60.3%
2.7%
FL2-H
FL1-H
Propidium iodide
Annexin V

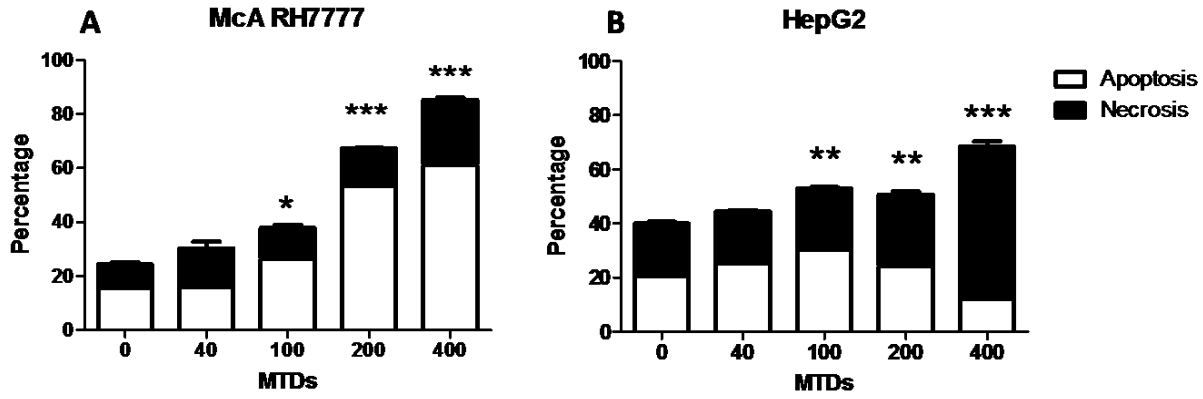


Figure 9. MTDs cause hepatocyte cell death in a dose-dependent manner *in vitro*.

A, MTDs (Mitochondrial lysate) induce significant rat hepatocyte cell death at concentrations above 100 μg/ml, McA RH7777 co-cultured with different concentrations of MTDs (μg/mL) for 24 hours;

B, MTDs induce significant human hepatocytes cell death at concentrations above 100 μg/ml, HepG2 co-cultured with different concentrations of MTDs for 24 hours (μg/ml).

The blank bar indicates Annexin V positive cells, the black bar indicates PI positive cells. *, P=0.01, **, P=0.001, ***, P=0.0001 (n=3/group).

Although most immune responses in the liver are driven by activation of non-parenchymal cells, several studies have shown that hepatocytes also express pattern recognition receptors, such as toll like receptors (TLRs), to sense danger signals [303-305]. I next determined whether TLRs expressed by hepatocytes play a role in response to extracellular mitochondrial components. Expression of mRNA for *TLR2*, *TLR4*, *TLR9*, *MyD88* and *NFκB* were similar for hepatocytes co-cultured with 400μg/ml MTDs, warm IR and cold IR treatments (Figure 10). The expression levels of all these genes were significantly higher in hepatocytes co-cultured with MTDs as well as the warm and cold IR treatments when compared to the control group. The expression levels, all normalized to the control group, are as follows, TLR2 (MTDs: 3.572±0.426, $p<0.01$; warm IR: 5.302±0.462, $p<0.001$; cold IR: 5.492±0.893, $p<0.001$), TLR4 (MTDs: 7.309±0.635, $p<0.0001$; warm IR: 7.953±0.794, $p<0.0001$; cold IR: 4.753±0.594, $p<0.001$), TLR9 (MTDs: 3.246±0.145, $p<0.001$; warm IR: 2.129±0.430, $p<0.01$; cold IR: 8.528±0.303, $p<0.0001$), MyD88 (MTDs: 16.04±1.718, $p<0.001$; warm IR: 19.90±4.494, $p<0.001$; cold IR: 6.744±0.977, $p<0.01$), and NFκB (MTDs: 7.591±0.802, $p<0.001$; warm IR: 7.589 ±1.266, $p<0.001$; cold IR: 4.176±0.970, $p<0.01$).

The pro-inflammatory cytokines, IL-6 and TNF, were also assayed in the supernatants by ELISA. IL-6 levels were not increased in the control, MTDs co-cultured or warm/cold IR groups (Data not shown). Interestingly, we found a small amount of TNF in the supernatant of both the MTDs co-cultured hepatocytes (20.23±6.971pg/ml) and the cells subjected to warm IR (6.799±3.519pg/ml), whereas these levels were undetectable in the control group and in cells subjected to cold IR (Table 2). We confirmed that the TNF expression level was significantly up-regulated in hepatocytes co-cultured with MTDs by

qPCR (Figure 11), which demonstrated a TNF mRNA expression level 16.12±2.974 fold higher than the control group ($P<0.001$).

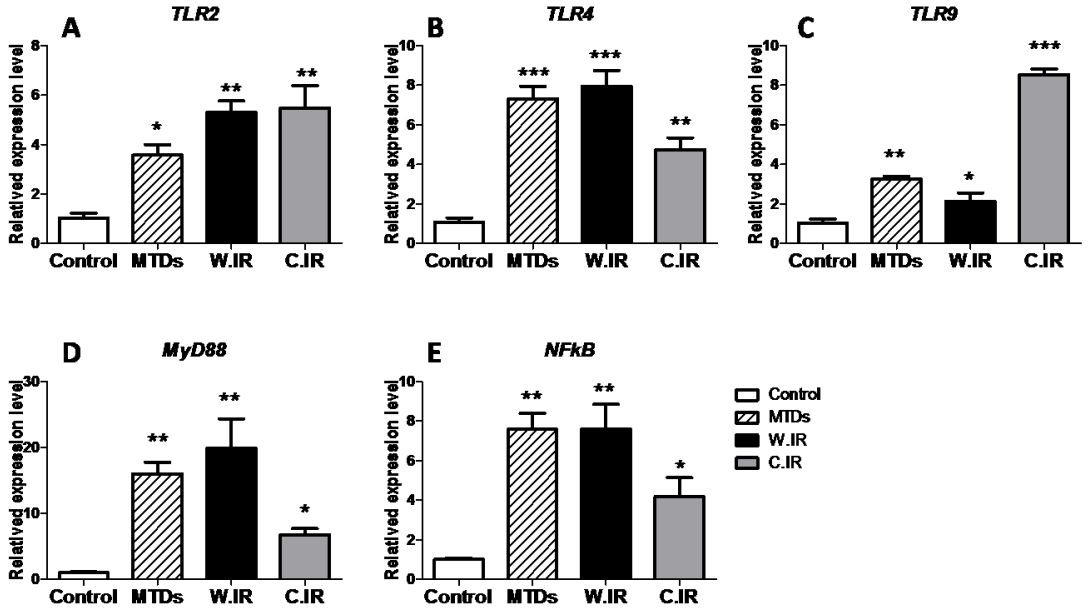


Figure 10. Fold changes in *TLR2*, *TLR4*, *TLR9*, *MyD88* and *NFκB* mRNA levels in response to MTDs and *IR in vitro*.

MTDs induce *MyD88* and *NFκB* expression and up-regulate expression of *TLR2*, *TLR4* and *TLR9* similar to the impacts of warm hepatic IR.

A-E, The expression level of *TLR2, TLR4, TLR9, MyD88* and *NFκB* significantly increased with MTDs co-culture, warm IR, and cold IR treatment compared to control; MTDs, co-culture MTDs with McA RH7777 hepatocyte cell line for 24 hours; W.IR, McA RH7777 hepatocyte cell line underwent warm ischemia for 1 hour followed by reperfusion for 24 hours *in vitro*; C.IR, McA RH7777 hepatocyte cell line underwent cold ischemia for 6 hours followed by reperfusion for 24 hours *in vitro*. *, P=0.01, **, P=0.001, ***, P=0.0001 (n=3/group)

Table 2. TNF released by hepatocytes co-cultured with mitochondrial lysate (MTDs) or subjected to warm IR.

	Control	MTDs*	Warm IR	Cold IR
TNFα	NA	20.23±6.971	6.799±3.519	NA

* MTDs, co-culture 400μg/ml MTDs with McA RH7777 hepatocyte cell line for 24 hours; W.IR, McA RH7777 hepatocyte cell line underwent warm ischemia for 1 hour followed by reperfusion for 24 hours *in vitro*; C.IR, McA RH7777 hepatocyte cell line underwent cold ischemia for 6 hours followed by reperfusion for 24 hours *in vitro*. NA, not available.

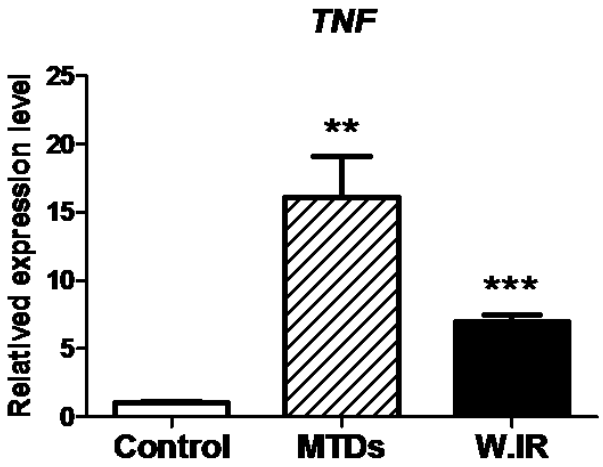


Figure 11. Fold changes in *TNF* mRNA levels in response to MTDs and IR *in vitro*. MTDs-induced hepatocytes up-regulate expression of TNF similar to warm IR treatment. MTDs, co-culture 400µg/ml MTDs with McA RH7777 hepatocyte cell line for 24 hours; W.IR, McA RH7777 hepatocyte cell line underwent warm ischemia for 1 hour and reperfusion for 24 hours *in vitro*. **, P=0.001, ***, P=0.0001 (n=3/group)

3.1.4 Circulating mitochondrial components are increased in rodent hepatic IR *in vivo*

To investigate whether these *in vitro* results could be replicated *in vivo*, levels of mtDNA in the blood of rats subjected to total hepatic 1h warm ischemia followed with 1h reperfusion *in situ* or sham group was assessed. In this *in vivo* model, the IR group displayed a significant increase in mtDNA over time, compared to the control group (Figure 12A). The mtDNA levels of the control group also increased at 30min but returned to baseline after 60min and remained stable during the next 60min (Figure 12B). This result indicated that total hepatic IR can elicit a significant increase in circulating mtDNA in blood in both ischemia and reperfusion phases.

Having demonstrated mtDNA release during in rat total hepatic IR1+1h model, whether mtDNA as mitochondrial components released markers were still detectable in a longer term of reperfusion was assessed. By using 70% hepatic 1h warm ischemia and reperfusion for one or six hours in mice which allowed animal to recover after hepatic IR surgery, I found that, similar to the rat total hepatic IR model (Figure 12 A and B), after one hour of reperfusion, there was significant mtDNA in the mouse plasma compared to sham and after six hours of reperfusion, the IR 1h+6h group still had a significant increase of mtDNA in the plasma than related sham group. Notably, a longer term (6h) after hepatic ischemia surgery enhanced mtDNA release in both sham and IR 1+6h groups compared to 1h after ischemia surgery (Figure 12C). Therefore, my data suggested that mtDNA can be released to blood circulation during hepatic IRI, and the elevation of mtDNA was detectable not only in an acute phase as 1h reperfusion but also a longer phase (6h) of reperfusion.

Figure 12. Hepatic IR increases mitochondrial DNA levels in circulation *in vivo*.

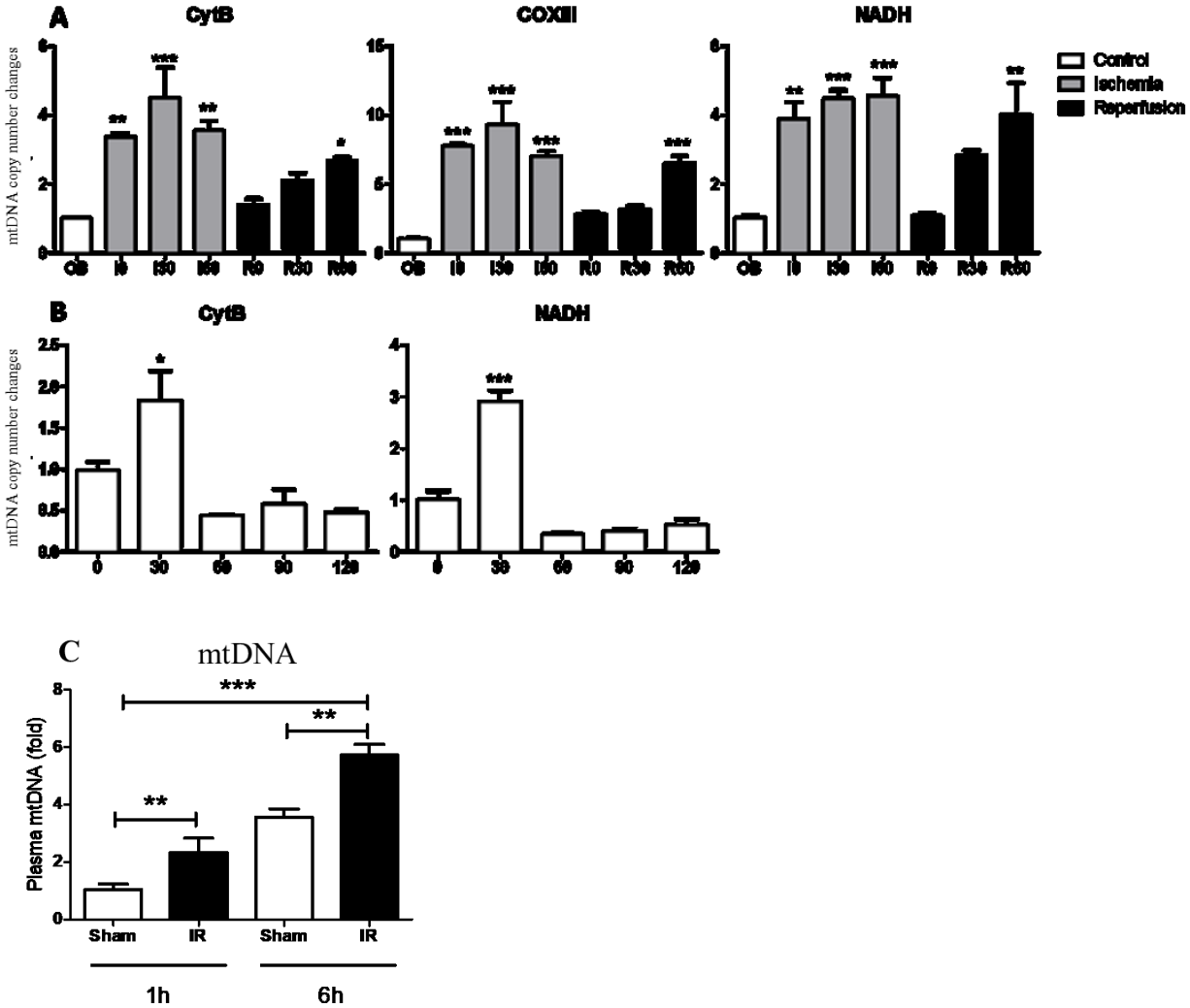
A, One hour of warm ischemia and one hour of warm reperfusion *in vivo* significantly increased the mtDNA levels during both ischemia and IR; OB, blood collection immediately following laparotomy; I, ischemia: the atraumatic clip was placed on the portal triad for 60 minutes; R, reperfusion; reperfusion was initiated by removal of the atraumatic clip and observed for another 60 minutes; I0, I30, I60: ischemia for 0/30/60 minutes; R0, R30, R60: after 60 minutes portal triad clamping and the clip was removed to achieve reperfusion for 0/30/60 minutes;

B, mtDNA levels were not altered after 2 hours following a sham operation. 0, 30, 60, 90, 120 indicate minutes of sham operating time, laparotomy without clamping of the portal triad.

C, 70% hepatic ischemia significantly induced mtDNA release into blood and a longer term of reperfusion enhanced mtDNA elevation.

*, P=0.01, **, P=0.001, ***, P=0.0001 (n=3/group)

Figure 12


A
CytB
COXIII
NADH
mtDNA copy number changes
Control
Ischemia
Reperfusion
B
CytB
NADH
mtDNA copy number changes
C
mtDNA
Plasma mtDNA (fold)
Sham
IR
Sham
IR
1h
6h

3.1.5 Hepatic IR triggers liver injury and inflammatory cytokines production *in vivo*

The impact of total hepatic IR on the injury of hepatocytes was examined *in vivo*. Significantly more liver injury in the IR treatment group was observed by histological analysis compared to sham animals (Figure 13 A and B). There were more necrotic cells with cell membrane disruption as indicated by the yellow arrows in Figure 13A. Liver enzyme levels remained within the normal range in the control, sham operated group for the duration of the study. A statistically significant difference was noted in mean serum ALT and AST levels following the induction of IR, compared with sham operated rats (Figure 14). Pro-caspase-3 is an intrinsic protein that is cleaved to segments when a cell undergoes apoptosis. Cleaved-caspase-3 is commonly used as an apoptotic marker [306]. Apoptosis was also assessed in these groups with cleaved-caspase-3/ pro-caspase-3 immunohistochemistry (IHC). Liver sections from the IR group were highly positively stained for the cleaved form of caspase-3 compared to the sham group (Figure 15). In addition, there was a trend that cleaved-caspase-3 was accumulated while pro-caspase-3 was reduced in the livers of the IR group, suggesting more apoptotic cells in these livers.

To determine whether these findings correlated with production of inflammatory cytokines following IR, ELISA assays were conducted to measure the serum levels of TNF, IL-6, and IL-10 in the sham and IR groups. Among the cytokines examined, IL-6 and IL-10 production were found to be markedly increased in the serum after just one hour of hepatic IR (Figure 16A and C), whereas TNF production showed a slight increase when compared to the sham group (Figure 16B). The increases in IL-6, TNF and IL-10 were associated with an increase in circulatory mitochondrial components.

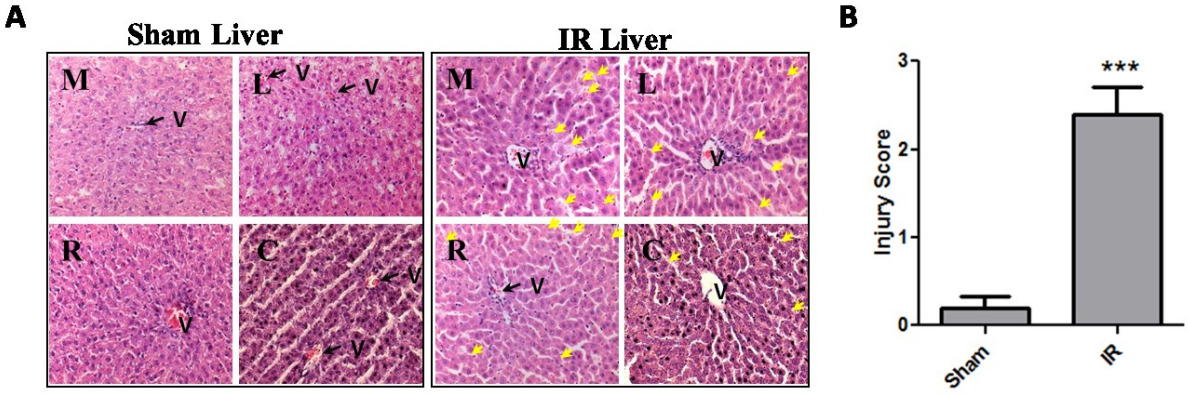


Figure 13. Histopathologic analyses of liver injury after IR.

Liver sections were obtained from IR and sham control rats at the time point of 120 minutes. A, IR induce hepatic injury assessed by H&E staining from different liver lobes: median (M), left (L), right (R) and caudate (C) lobes of sham and IR group, black arrows with V's point to the portal vein, yellow arrows indicate necrotic cells; B, IR induce significant hepatic injury assessed by Suzuki injury score [307]. ***, P=0.0001(n=3/group)

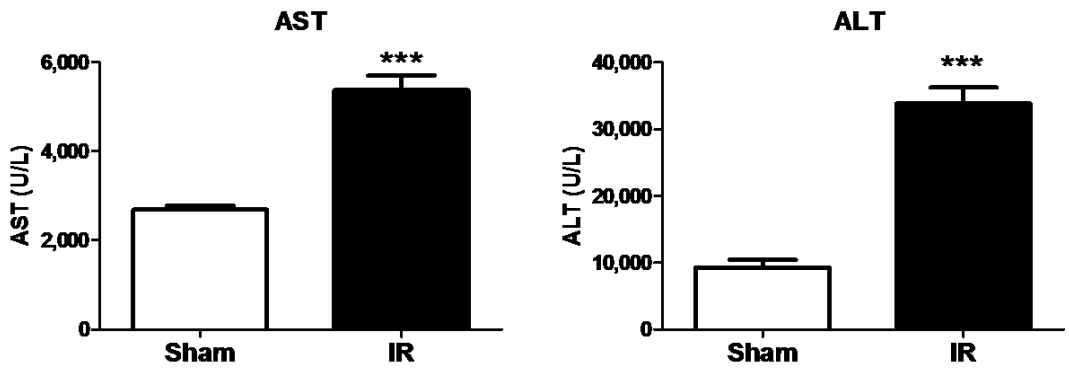


Figure 14. Hepatocellular injury evaluated by AST and ALT.

IR induce significant hepatic injury as demonstrated by elevated AST and ALT levels. Serum samples were obtained from IR and sham rats at 120 minutes post-reperfusion. A statistically significant difference was noted in mean serum ALT and AST levels following the induction of IR, compared with sham operated rats *in vivo*. ***, P=0.0001(n=3/group)

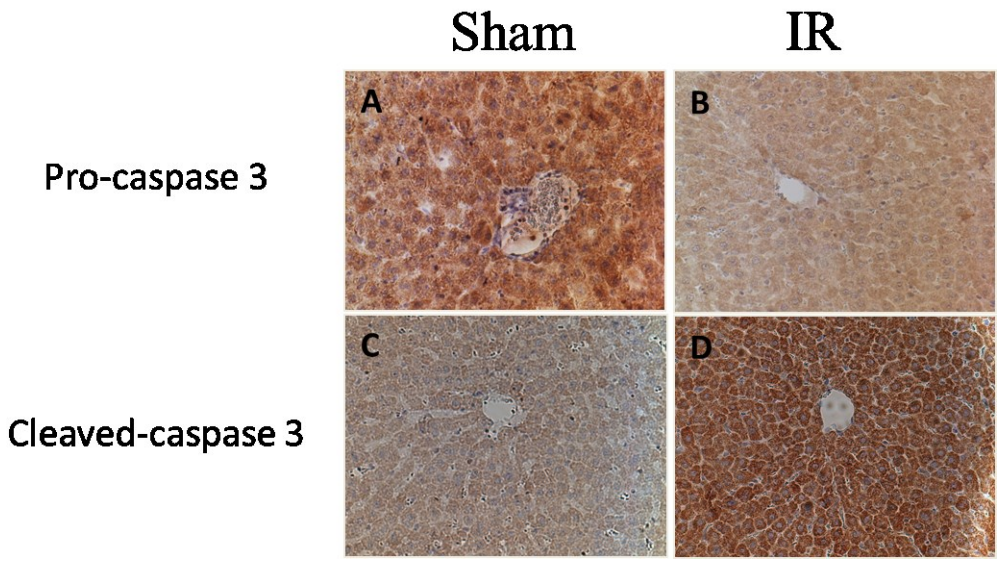


Figure 15. Total hepatic IR causes apoptosis *in vivo*.

Liver sections were obtained from IR and sham control rats at 120 minutes post-reperfusion. A and B, the intensity of immunostaining of pro-caspase 3 significantly decreased after IRI compared with the sham group; C and D, the intensity of immunostaining of cleaved-caspase 3 significantly increased after IRI compared with the sham group.

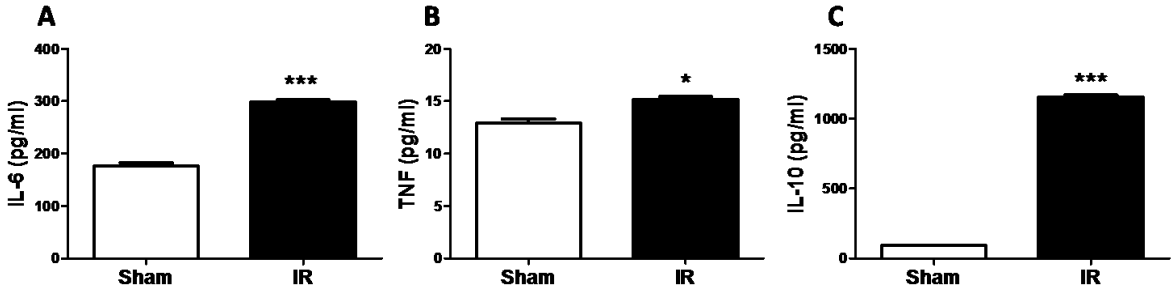


Figure 16. Total hepatic IR cause systemic inflammation in *in vivo*.

Total hepatic IR induces significant IL-6 (A), TNF (B), and IL-10 (C) release. Serum samples were obtained from IR and sham rats at 120 minutes post-reperfusion. *, P=0.01, ***, P=0.0001 (n=3/group).

3.1.6 Hepatic IR induces mitochondrial damage and mitochondria release to extracellular milieu

To further confirm mitochondrial damage in liver during hepatic IRI, liver biopsies were collected from both IR and sham livers and mitochondrial morphology was assessed by transmission electron microscopy (TEM). Overall, in sham control groups, mitochondria were electron dense (dark color) randomly located in a clear background cytosol with integrated cristae structure (Figure 17 A, E and I), whereas in the biopsy of IR liver, large numbers of mitochondria were observed located towards the edge of hepatocytes (red arrows, Figure 17B and F) and the background of cytosol became darker while the electron density of mitochondria was reduced (Figure 17J) when compared with sham control (Figure 17I). Surprisingly, FM were found in the extracellular milieu in both 1h reperfusion and 6h reperfusion livers (green heads, Figure 17B, K and L); some mitochondria in the IR liver lost their double membrane structure (yellow heads, Figure 17C and D) or changed their homeostasis as indicated by enlargement of mitochondrial size (>2μm, yellow heads in Figure 17H). Moreover, unlike mitochondria in sham group, they became less electron dense in the IR liver with evidence of cristae structure disruption (Figure 17H). Notably, more adherent immune cells were observed in the IR liver (yellow arrows, Figure 17F and G) than sham control. Furthermore, in Figure 17F and consistent with previous histology data, necrotic cells with cell membrane disruption were found in the IR liver. An interesting phenomenon was observed that immune cells (yellow arrows) were associated with areas of necrotic cells with liberated intact mitochondria, consistent with the possibility that extracellular mitochondria might have chemotactic functions.

Collectively, TEM helped to visualize and further confirm mitochondrial damage and liberation to the extracellular milieu in IR liver.

Figure 17. Hepatic IRI promote mitochondria damage and release *in vivo*.

A, Sham liver biopsy set as control for IR 1h+1h.

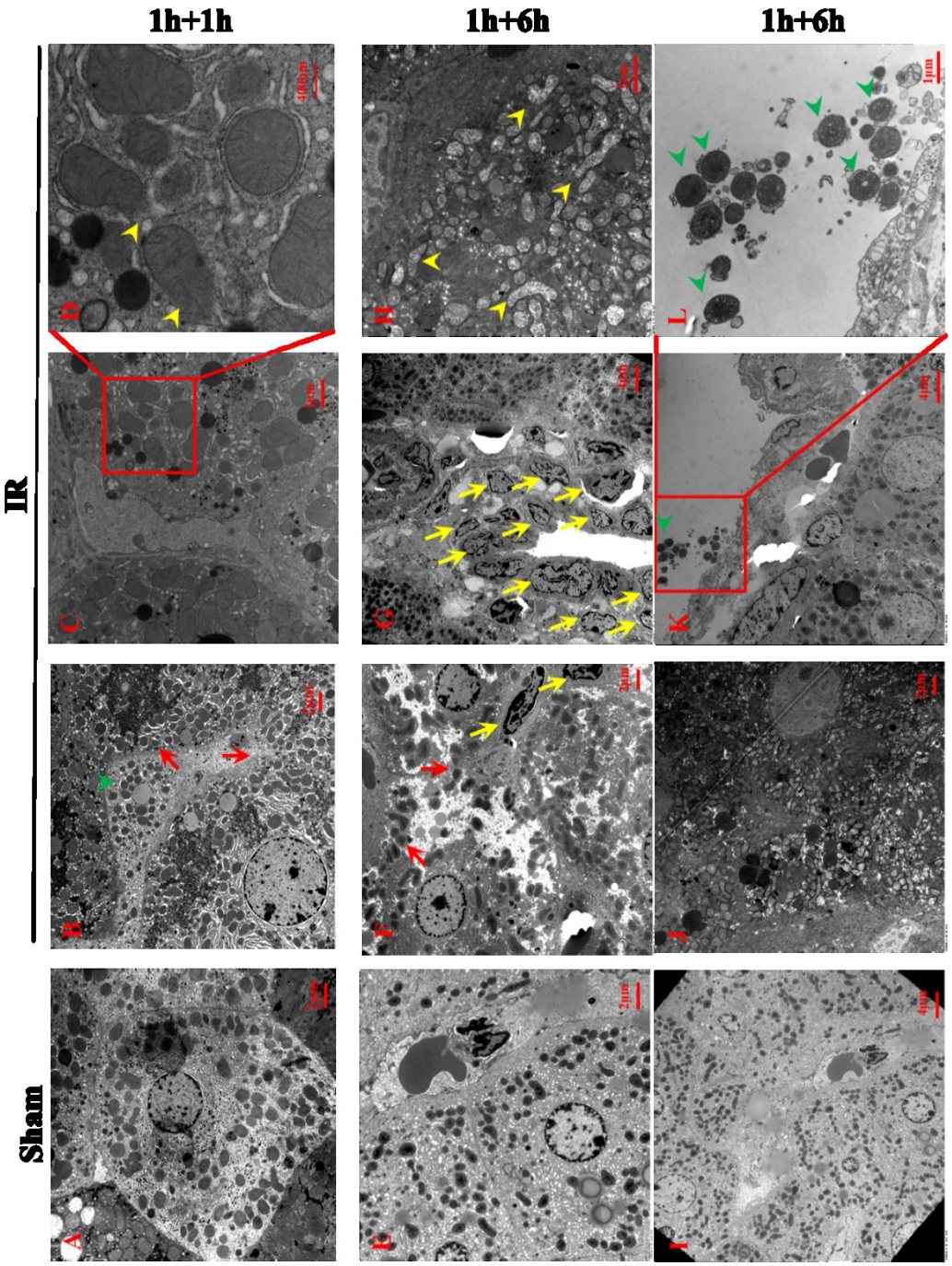
B-D, IR 1h+1h liver.

E and I, Sham liver biopsy set as control for IR 1h+6h.

F-H and J-L, IR 1h+6h liver.

TEM visualization of mitochondria (dark dots) located to the cell edge of hepatocytes (red arrows), free extracellular mitochondria released (green heads), aberrant mitochondrial homeostasis (mitochondrial membrane disrupted, or mitochondrial enlargement or cristae disrupted; yellow heads) and immune cell accumulation (yellow arrows) in the IR liver.

Figure 17



3.2 Extracellular mitochondria induce inflammation via a MC and formyl peptide receptor 1 (FPR1)-dependent manner

Mitochondria can act as alarmins and, as demonstrated in the previous section, were released and elevated in hepatic IRI both *in vitro* and *in vivo*. We therefore questioned whether extracellular mitochondria could further induce inflammatory responses. As adherent immune cells associated with areas of FM were visualized by TEM in Figure 17F, it was possible that extracellular mitochondria have chemo-attractive ability to recruit immune cells to a primary local site of mitochondrial release (or injection).

A pilot study was performed using an intravenous injection of mitochondria (100 μg/ml equal to the mitochondrial released by a 5% liver injury) through the tail vein to check if extracellular mitochondria could worsen hepatic IRI by recruiting inflammatory leukocytes. However, this model related to increasing the systematic circulation of mitochondrial components and thereby inducing inflammation rather than investigating the local liver environment. The injected mitochondrial components were likely captured by lung vasculature as indicated by significant increases of mtDNA in lung tissue one day after injection (Figure 18A). Furthermore, the lung was more susceptible to circulating mitochondrial alarmins as indicated by a significant increase in lung/body weight ratio but no change for liver/body weight ratio (Figure 18B) and marked inflammatory lung injury shown by haematoxylin and eosin histology (Figure 18C) compared to PBS injected controls.

To explore the influence of extracellular mitochondria to the local immune environment, a model using intraperitoneal injections of mitochondria was used in our following studies. This site allows for easy assessment of cell recruitment responses to

mitochondria and other alarmins and also for evaluation of local cytokine and chemokine responses at the protein level.

Figure 18. Circulating extracellular mitochondrial components induced lung injury *in vivo*.

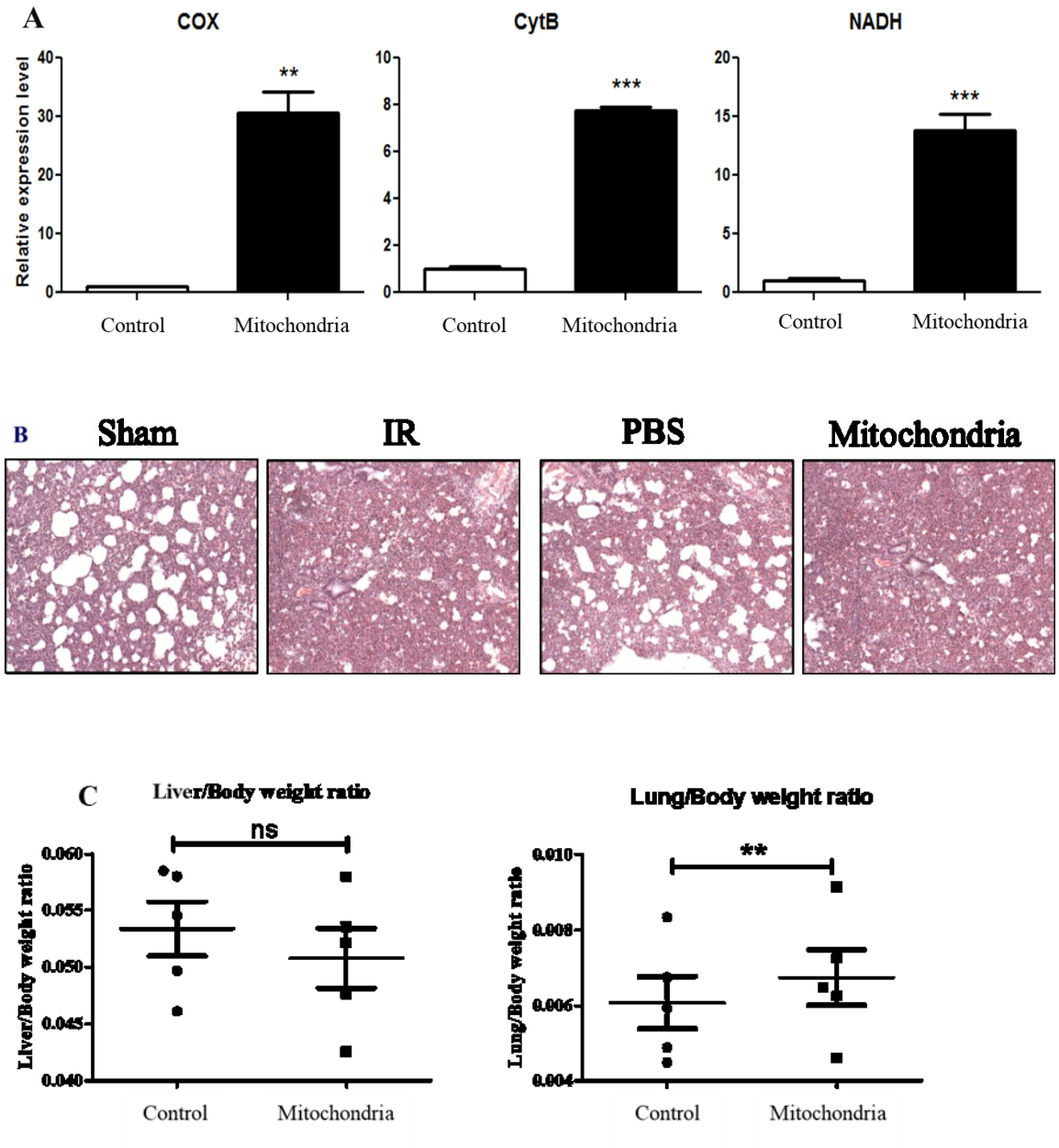
A, mtDNA levels were significantly elevated in lung tissue after one day of MTDs injection.

B, H&E staining for lung tissues from sham, total hepatic IR, PBS injection i.v. and MTDs injection groups.

C, liver/body weight ratio and lung/body weight ration compared between PBS control and MTDs i.v. injection groups.

All values are means ± SEM of 5 independent experiments. *, $P<0.01$, **, $P<0.001$, ***, $P<0.0001$.

Figure 18


A
COX
CytB
NADH
Relative expression level
40
30
20
10
0
**
10
8
6
4
2
0
***
20
15
10
5
0
***
Control
Mitochondria
Control
Mitochondria
Control
Mitochondria
B
Sham
IR
PBS
Mitochondria
C
Liver/Body weight ratio
ns
Liver/Body weight ratio
0.060
0.055
0.050
0.045
0.040
Control
Mitochondria
Lung/Body weight ratio
**
Lung/Body weight ratio
0.010
0.008
0.006
0.004
Control
Mitochondria

3.2.1. FM trigger peritoneal inflammation

Because neutrophils are critical early mediators of the innate immune response, we first sought to determine if FM would induce neutrophil recruitment to the site of injection to mimic the clinical scenario of how released FM during tissue injury activate the innate immune system. We used different concentrations of liver-derived FM (1, 2, 4, 6, 12 mg/kg) to model traumatic necrosis of 0.5%-6% of the mouse's liver [82]. Peritonitis induced by intraperitoneal injection of FM, indicated in an accumulation of neutrophils in the peritoneal lavage fluid, as defined by flow cytometry for $CD11b^{+}F4/80^{-}Ly6G^{+}$cells within 16 h. The peritoneal contents were harvested by lavage, and the total peritoneal cavity cells (PCC) were counted. We observed that the percentage and overall number of peritoneal neutrophils were increased in a dose-dependent manner following i.p. injection of mice with FM (Table 3), confirming that FM can activate cell recruitment.

To determine if FM could influence other immune cells when administered at clinically relevant levels, a dose of 5mg/kg FM in mice was injected i.p. which is equivalent to mitochondria from a 2.5% liver injury [82]. A comprehensive analysis of leukocyte recruitment was performed by flow cytometry (PBS n=11; FM n=10). The gating strategy used to identify cell populations in the mouse peritoneum is shown in Figure 19. The peritoneal cell numbers were significantly increased after FM (5mg/kg) injection (Figure 20A). The numbers of neutrophils ($CD11b^{+}F4/80^{-}Ly6G^{+}$), inflammatory macrophages (Inflamm macs, $CD11b^{+}Ly6C^{+}Ly6G^{-}$), DC ($CD11c^{+}MHCII^{+}$), plasmacytoid dendritic cells (pDC, $CD11c^{+}MHCII^{+}CD317^{+}B220^{+}$), tolerogenic DC (TDC, $CD11c^{+}MHCII^{+}CD103^{+}$), MC ($CD117^{+}Fc\varepsilon RI^{+}$), eosinophils ($Siglec\text{-}F^{+}CD11b^{-/lo}$), and NK cells ($CD3^{-}CD19^{-}NK1.1^{+}$) were all significant elevated (Figure 20B, D-I). Meanwhile T

helper cells ($CD3^+CD19^-CD4^+CD8^-$), cytotoxic T cells ($CD3^+CD19^-CD4^-CD8^+$), $CD11b^+$ B cells ($CD3^-CD19^+CD11b^+$), and $CD11b^-$ B cells ($CD3^-CD19^+CD11b^-$) stayed within a normal range at the 16h timepoint (Figure 20 J-N). In contrast, resting macrophages (resting Macs, $CD11b^+F4/80^{hi}Ly6G^-$) were significantly decreased after FM injection (Figure 20C). Representative FACS analysis data illustrate the myeloid cell populations for both PBS (Figure 20O) and FM (Figure 20P) injected C57BL/6 mice. In addition, following injection with FM, DC up-regulated expression of the early activation marker CD69 and co-stimulation molecule CD24 (Figure 20O and P). Overall, FM were observed to induce a complex, predominantly neutrophilic acute inflammatory response in the peritoneal cavity.

Table 3. FM induce neutrophil recruitment in a dose-dependent manner.

C57BL/6 mice given intraperitoneally FM (1-12mg/kg) exhibit marked evidence of neutrophil recruitment. After 16 hour the peritoneal contents were harvested by lavage, the total peritoneal cavity cells (PCC) were counted, and the percentage of neutrophils ($CD11b^+$ $Ly6C^+Ly6G^+$) in each sample were identified by flow cytometric analysis. The actual neutrophil numbers were calculated based on the percentage of neutrophils and the total number of PCC counting. The graphs shown are the mean±SEM. n=4 per group; ns=not statistically significant, *, P< 0.05; ***, P<0.0005; ****, P< 0.0001.

	PBS	**Free mitochondria**				
		1mg/kg	2mg/kg	4mg/kg	6mg/kg	12mg/kg
Percentage	0.6875±0.09411	1.458±0.3895^{ns}	2.998±0.3253^{ns}	5.283±0.5841*	15.95±1.044****	33.80±1.592****
Numbers	14546±489.0	17567±1975^{ns}	83438±2433^{ns}	177000±36494*	405696±68218****	645173±25697****

Figure 19. Gating strategy used to identify cell population in the mouse peritoneum. Cells were harvested from the peritoneal cavity by lavage, counted, centrifuged, and incubated in 5% FBS on ice prior to staining with specific antibodies. Staining is shown for a PBS injected mouse at 16h. After an initial FSC/SSC scatter gate, a sequential gating strategy was used to identify populations expressing specific markers: neutrophils ($CD11b^{+}$ $F4/80^{-}Ly6G^{+}$), resting macrophages (resting Macs, $CD11b^{+}F4/80^{hi}Ly6G^{-}$), inflammatory macrophages (Inflamm macs, $CD11b^{+}Ly6C^{+}Ly6G^{-}$), eosinophils (Siglec-$F^{+}CD11b^{-/lo}$), DC ($CD11c^{+}MHCII^{+}$), pDC ($CD11c^{+}MHCII^{+}CD317^{+}B220^{+}$), TDC ($CD11c^{+}MHCII^{+}CD103^{+}$), MC ($CD117^{+}Fc\varepsilon RI^{+}$), T helper cells ($CD3^{+}CD19^{-}CD4^{+}CD8^{-}$), cytotoxic T cells ($CD3^{+}CD19^{-}CD4^{-}CD8^{+}$), NK cells ($CD3^{-}CD19^{-}NK1.1^{+}$), $CD11b^{+}$ B cells ($CD3^{-}$ $CD19^{+}CD11b^{+}$), and $CD11b^{-}$ B cells ($CD3^{-}CD19^{+}CD11b^{-}$). FSC, forward scatter; MHCII, major histocompatibility complex class II; SSC, side scatter.

Figure 19

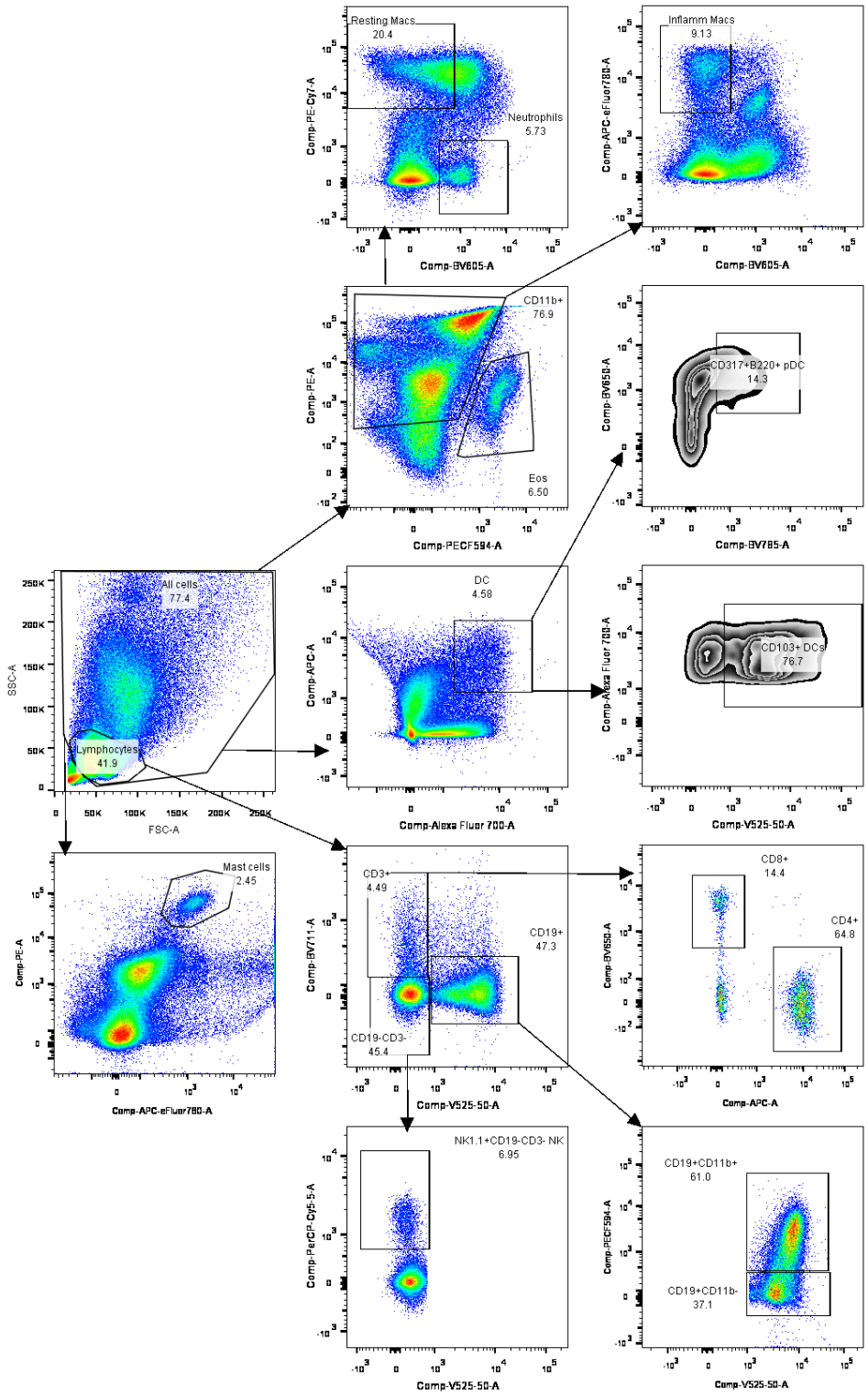

Resting Macs
20.4
Neutrophils
5.73
Comp-PE-Cy7-A
Comp-BV605-A
Inflamm Macs
9.13
Comp-APC-eFluor780-A
Comp-BV605-A
CD11b+
76.9
Eos
6.50
Comp-PE-A
Comp-PECF594-A
CD317+B220+ pDC
14.3
Comp-BV650-A
Comp-BV785-A
All cells
77.4
Lymphocytes
41.9
SSC-A
FSC-A
DC
4.58
Comp-APC-A
Comp-Alexa Fluor 700-A
CD103+ DCs
76.7
Comp-Alexa Fluor 700-A
Comp-V525-50-A
Mast cells
2.45
Comp-PE-A
Comp-APC-eFluor780-A
CD3+
4.49
CD19+
47.3
CD19-CD3-
45.4
Comp-BV711-A
Comp-V525-50-A
CD8+
14.4
CD4+
64.8
Comp-BV650-A
Comp-APC-A
NK1.1+CD19-CD3- NK
6.95
Comp-PerCP-Cy5-5-A
Comp-V525-50-A
CD19+CD11b+
61.0
CD19+CD11b-
37.1
Comp-PECF594-A
Comp-V525-50-A

Figure 20. FM induce peritoneal inflammation.

C57BL/6 mice injected i.p. with FM (5mg/kg) equivalent to mitochondria from a 2.5% liver injury exhibit marked evidence of leukocyte recruitment. Peritoneal contents were harvested by lavage after 16 hours, (A) the total peritoneal cavity cells (PCC) were counted, and the percentage of leukocytes in each sample was identified by flow cytometric analysis. (B) Neutrophils, (C) Resting macrophages, (D) Inflammatory macrophages, (E) Dendritic cells, (F) Plasmacytoid dendritic cells (pDC), (G) Tolerogenic dendritic cells (TDC), (H) MC, (I) Eosinophils, (J) Natural killer cells (NK), (K) CD3+ T cells, (L) CD4+ T helper cells, (M) CD8+ Cytotoxic T cells, (N) B cells. (O) and (P) Staining for the phenotype of myeloid cells for both PBS and FM injected C57BL/6 mouse. The graphs shown are the mean±SEM, PBS group n=11; FM group n=10. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 20

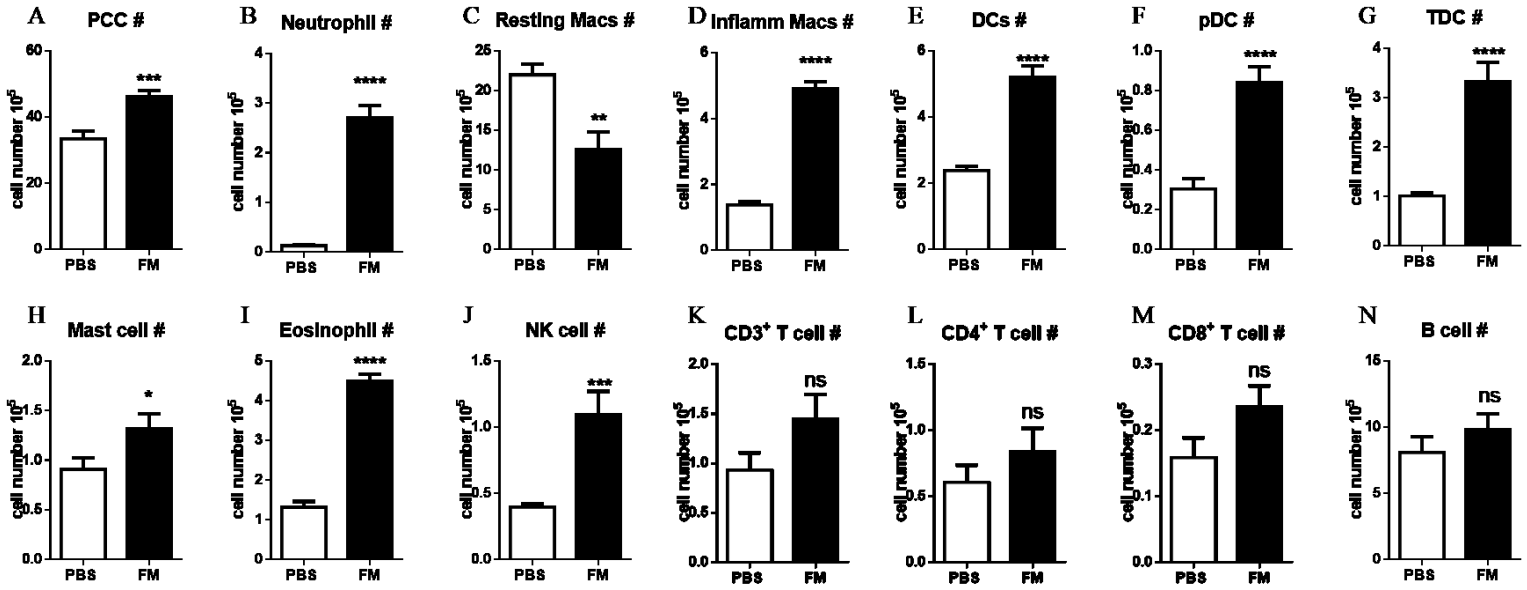
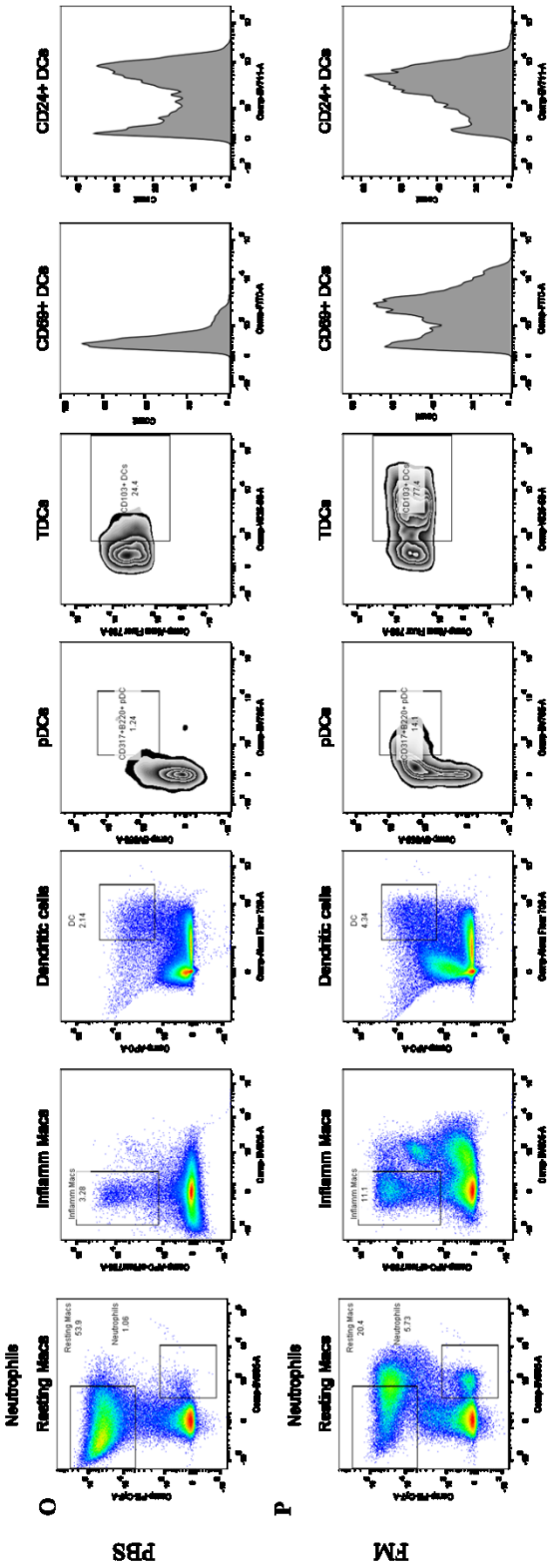

A PCC #
B Neutrophil #
C Resting Macs #
D Inflamm Macs #
E DCs #
F pDC #
G TDC #
H Mast cell #
I Eosinophil #
J NK cell #
K CD3+ T cell #
L CD4+ T cell #
M CD8+ T cell #
N B cell #
cell number 10^5
PBS
FM
***
****
**
*
ns

Figure 20 continued



3.2.2. MC play important roles in leukocyte influx *in vivo*

MC have been well-characterized in terms of their role in allergic reactions induced by crosslinking their surface immunoglobulin E [265, 308, 309]. However, they are also associated with innate immunity and with leukocyte recruitment following exposure to microbial pathogens [258, 310-312] or endogenous danger signals [313-316]. The role of MC in FM induced leukocyte influx was therefore examined. FM or diluent (PBS) was injected i.p. into MC competent C57BL/6 mice (B6) and MC deficient C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) mice. As shown in Figure 20, FM induced myeloid cell influx into the peritoneal cavity at 16h. The distributions of the immune lineages (including resting macrophages (resting Macs, CD11b^{+}F4/80^{hi}Ly6G^{-}), inflammatory macrophages (Inflamm macs, CD11b^{+}Ly6C^{+}Ly6G^{-}), neutrophils (CD11b^{+}F4/80^{-}Ly6G^{+}), DC (CD11c^{+}MHCII^{+}), plasmacytoid dendritic cells (pDC, CD11c^{+}MHCII^{+}CD317^{+}B220^{+}), tolerogenic DC (TDC, CD11c^{+}MHCII^{+}CD103^{+}), and eosinophils (Siglec-F^{+}CD11b$^{-/lo}$)) from B6 mice versus Wsh mice were presented as two-dimensional (2D) t-distributed stochastic neighbor embedding (t-SNE) plots (Figure 21A-D). The percentages of each immune lineage were manually gated using FlowJo in each individual PCC (Appendix 1), which validated the significant enrichment of neutrophils (Appendices 1B), DC (Appendices 1E), and eosinophils (Appendices 1J) in PCC from wild type but not Wsh FM-injected mice. To aid with comparison of these two mouse strains to further explore the role of MC involved in the FM induced leukocyte influx, we compared the cell number fold changes for all four groups (Figure 21E-Q). In Figure 21E, the increase in PCC number induced by FM injection was significant lower in the Wsh mice than the B6 mice. Of note, accumulation of neutrophils, DC, pDC, TDC, and eosinophils were significantly reduced in the Wsh MC deficient mice

(Figure 21F, I-L). Whereas, the other major lineages showed no significant differences between these two mouse strains (Figure 21H, M-Q, t-SNE plot data not shown). Representative plots show the percentages of neutrophils, macrophages, DC and expression of CD69 and CD24 in PBS injected and FM-injected Wsh mice in Figure 21R and 21S. These data suggest that MC may play an important role in myeloid cell recruitment, particularly of neutrophils and DC.

Figure 21. Comparison of FM induced peritoneal inflammation between C57BL/6 (B6) wildtype mice and C57BL/6-KitW-sh (Wsh) MC deficient mice.

Wsh mice showed a reduced FM induced myeloid cell recruitment. B6 and Wsh mice were injected i.p. with PBS or FM (5mg/kg). After 16 hours the peritoneal contents were harvested by lavage, total peritoneal cavity cells (PCC) were counted, and the percentage of leukocytes in each sample were identified by flow cytometric analysis. t-SNE was performed to analyze the myeloid cells on B6 PBS (A), B6 FM (B), Wsh PBS (C), Wsh FM (D) mouse. The indicated cellular subsets (colored legends) were manually gated and overlaid onto the total live cells (pink). The clusters were overlaid with the subset population color. (E) The fold changes of PCC number induced by FM injection compared to PBS injection, the white bar represents the cell number fold change in C57BL/6 (B6) wildtype mice, the black bar represents the cell number fold change in C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) MC deficient mice. Cell number fold changes in (F)Neutrophils , (G) Resting macrophages, (H) Inflammatory macrophages, (I) Dendritic cells, (J) Plasmacytoid dendritic cells (pDC), (K) Tolerogenic dendritic cells (TDC), (L) Eosinophils, (M) Natural killer cells (NK), (N) CD3^{+} T cells, (O) CD4^{+} T helper cells, (P) CD8+ Cytotoxic T cells, and (Q) B cells. Representative plots are shown for the phenotype of myeloid cells for both PBS (R) and FM (S) injected C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) MC deficient mice. The graphs shown are the mean±SEM, n=5- 11. *, P< 0.05; **, P<0.01; ****, P< 0.0001

Figure 21

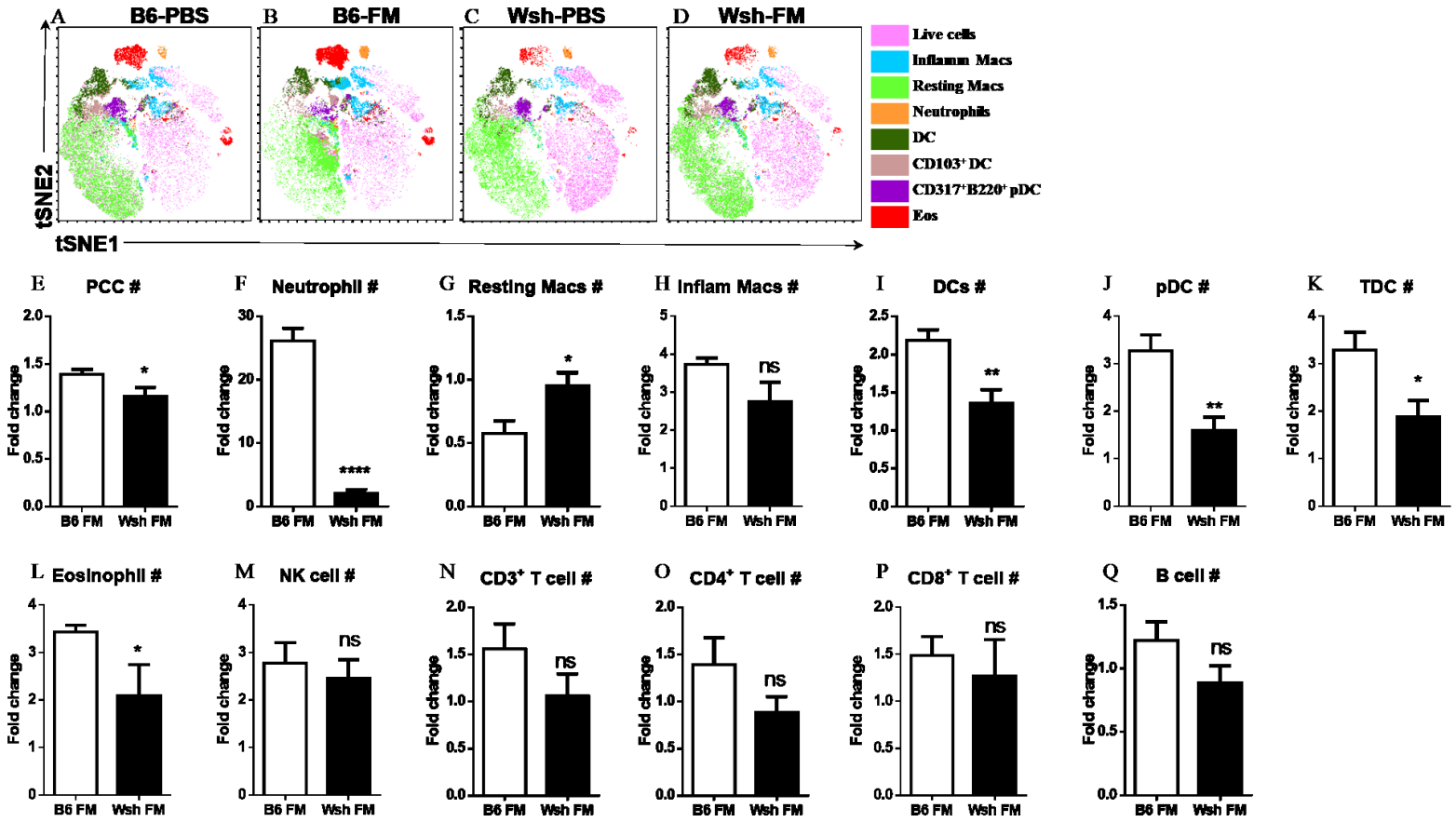
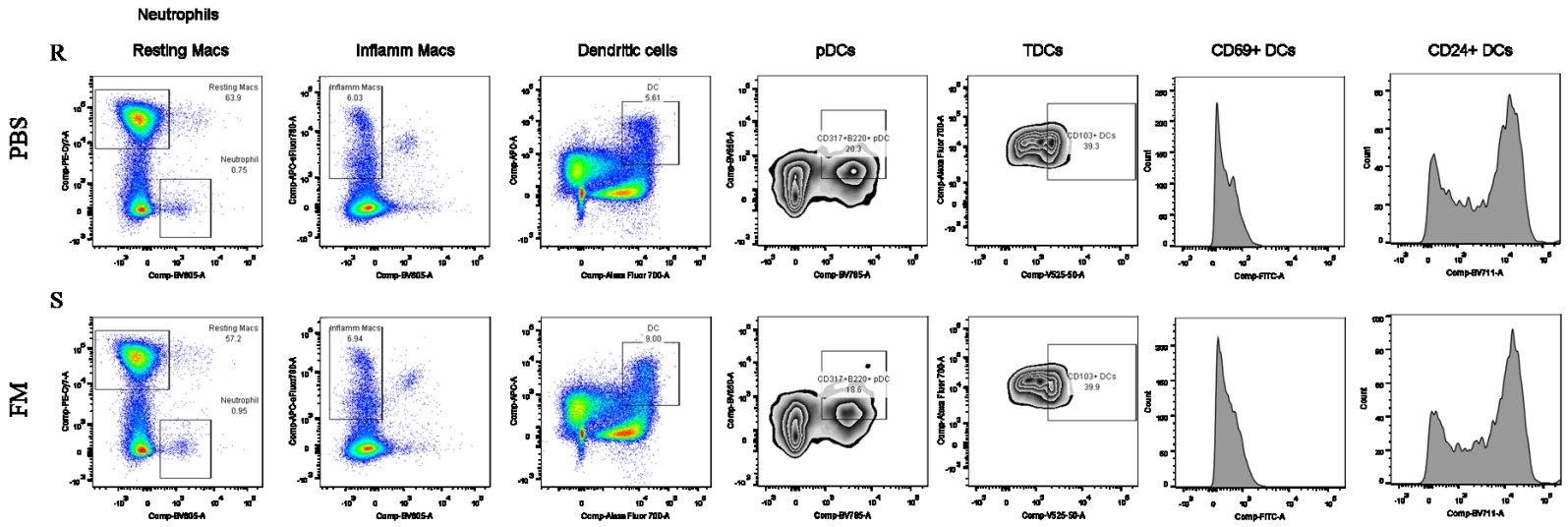

A B6-PBS
B B6-FM
C Wsh-PBS
D Wsh-FM
tSNE2
tSNE1
Live cells
Inflamm Macs
Resting Macs
Neutrophils
DC
CD103+ DC
CD317+B220+ pDC
Eos
E PCC #
F Neutrophil #
G Resting Macs #
H Inflam Macs #
I DCs #
J pDC #
K TDC #
L Eosinophil #
M NK cell #
N CD3+ T cell #
O CD4+ T cell #
P CD8+ T cell #
Q B cell #
Fold change
B6 FM
Wsh FM
*
****
ns
**

Figure 21 continued



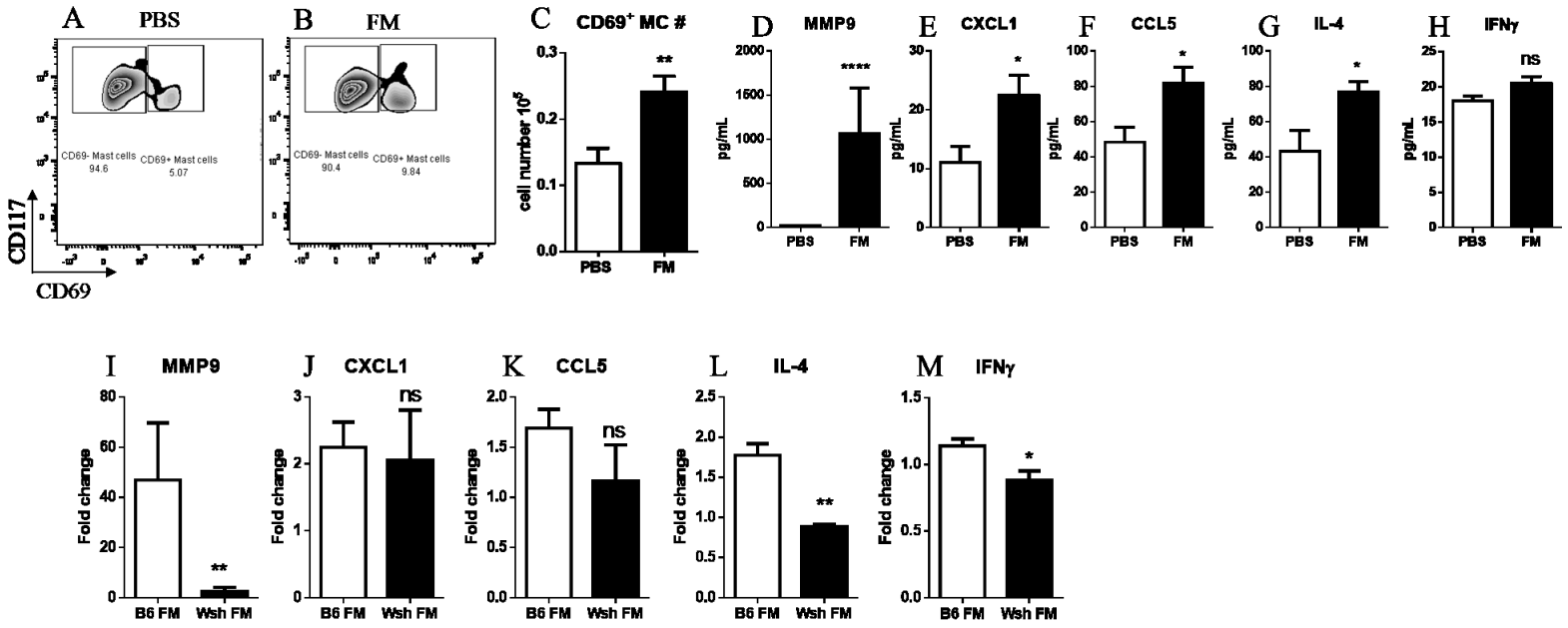
3.2.3. MC respond to FM *in vivo*

MC have the ability to relay local inflammatory signals to distant target cells and tissues by activation that results in the production of many inflammatory mediators [317]. We hypothesized that FM present in peritoneal cavity can activate MC to produce increased levels of inflammatory mediators. Following injection of FM, wild type mice had a significantly increased percentage and number of activated MC ($CD117^{+}Fc\varepsilon RI^{+}CD69^{+}$) when compared with PBS control mice (Figure 22A-C). Eighteen prominent inflammatory mediators, which could be secreted by MC upon activation [317], were assessed in peritoneal lavage using a multiplex protein assay. While several of these mediators were below the level of detection of the assay, mice injected with FM had significantly higher levels of MMP9, CXCL1, CCL5 and IL-4 (Figure 22D-H). A small numerical increase in IFNγ was not statistically significant (Figure 22H). To determine the role of MC, protein mediator responses were compared in peritoneal fluid obtained from Wsh MC deficient mice and wild type mice. The MMP9 response to FM challenge was significant reduced in the Wsh mice (Figure 22I). Wsh mice also did not demonstrate an increase in local IL-4 in response to FM and had reduced levels of IFN following FM challenge (Figure 22I-M). Lavage was markedly less inflammatory in the Wsh mice than B6 mice. Significant differences in MMP9, IL-4 and IFNγ production were observed for Wsh mice lavage samples compared with B6 mice lavage samples (Figure 22I, L and M). Similar CXCL1 and CCL5 responses were observed in the Wsh mice compared to B6 mice (Figure 22J and K). Collectively, these data indicate that MC are activated following FM injection and contribute to inflammatory mediator production in FM triggered peritonitis.

Figure 22. FM induce MC activation *in vivo*.

The phenotype of MC in mouse peritoneum, the activated MC are $CD69^+$ MC. (A) C57BL/6 mouse inject with PBS, (B) C57BL/6 mouse injected with FM (5mg/kg), (C) Numerical changes of $CD69^+$ MC. The cytokines and chemokines secretion in the mouse peritoneal lavage, (D) MMP9, (E) CXCL1, (F) CCL5, (G) IL-4, (H) IFNγ. Fold changes in the FM injected lavage between C57BL/6 (B6) wildtype mice and C57BL/6-$Kit^{W\text{-}sh}$ (Wsh) MC deficient mice are shown (I-M). The graphs shown are the mean±SEM, n=5-11. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$.

Figure 22


A PBS
B FM
CD69- Mast cells 94.6
CD69+ Mast cells 5.07
CD69- Mast cells 90.4
CD69+ Mast cells 9.84
CD117
CD69
C CD69+ MC #
cell number 10^5
D MMP9
E CXCL1
F CCL5
G IL-4
H IFNγ
pg/mL
PBS
FM
I MMP9
J CXCL1
K CCL5
L IL-4
M IFNγ
Fold change
B6 FM
Wsh FM
ns

3.2.4. FM activate human and murine primary MC *in vitro*

Upon appropriate stimulation, MC can release newly-synthetized inflammatory mediators such as cytokines and chemokines [263]. We sought to determine whether MC would respond directly to FM in this way. Human FM were isolated from a human hepatocyte cell line. Human CBMC were incubated with FM, mitochondrial protein preparations or mitochondrial DNA. Multiplex protein assays of supernatants were used to profile the inflammatory mediators produced by MC at 6h and 24h co-culture. Data from all 21 cytokines and chemokines assessed are summarized in Table 4. MC produced significant levels of CCL2, CCL3, CCL4, MMP9, CXCL1, CXCL8, IL-6, VEGF-A, IL-1ra and IL-13 in response to FM at the 24h timepoint but did not demonstrate significant IL-1β or IFNγ responses (Figure 23). The secretion of CCL4, VEGF-A, IL-1ra and IL-13 was significantly induced by co-culture with FM as early as 6h (Figure 23C, H, I and K). When examining dose response data for mediator production we observed two different patterns. A 'bell-shaped' response curve was observed for FM induced CCL2, CCL3, CCL4, MMP9, CXCL1, CXCL8 and IL-6 (Figure 23A-G); while VEGF-A, IL-1ra and IL-13 (Figure 23H, I and K) showed a more direct dose dependency even at high concentrations. A similar increased pattern of VEGF-A, IL-1ra and IL-13 was detected when CBMC were incubated with mitochondrial protein lysate (Figure 24), which contain mitochondrial N-formylated peptides and have been considered as mitochondrial lysate (MTDs, previous roughly named as mitochondrial damage associated molecular patterns) in previous literature [82, 299]. mtDNA only induced increase in IL-1β (mock, 5.767 ± 1.356 pg/ml; mtDNA 20μg/ml, 11.69 ± 1.900; p= 0.0641) and CCL3 (mock, 350.8 ± 205.9 pg/ml; mtDNA 20μg/ml, 782.9 ± 123.8; p= 0.1464) at the 6h timepoint. Overall, these

results demonstrated that upon FM stimulation, human CBMC can be activated and release a large array of inflammatory mediators through potentially more than one activation mechanism.

Table 4. Alteration of inflammatory mediator production by human CBMC following activation triggered by different mitochondrial alarmins.

	6 HOURS	6 HOURS	6 HOURS	24 HOURS	24 HOURS	24 HOURS
	FM	**MTD**	**mtDNA**	**FM**	**MTD**	**mtDNA**
CXCL1	OOR <	OOR <	OOR <	↗	↓	↓
IL-1 BETA	↓	↓	↗	↓	↓	←→
IL-1RA	↗	↗	←→	↗	↗	←→
IL-6	↗	↘	←→	↗	↘	←→
CCL2	←→	←→	←→	↗	↘	←→
MMP9	←→	←→	←→	↗	↘	←→
TNF	←→	←→	←→	←→	↘	←→
IFN-GAMMA	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
IL-10	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
IL-5	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
CXCL8	↗	↘	←→	↗	↘	←→
CCL4	↗	↗	←→	↗	←→	←→
CCL5	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
VEGF-A	↗	↗	←→	↗	↗	←→
CCL11	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
IL-12 P70	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
IL-18	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
CCL3	←→	←→	↗	↗	←→	←→
GM-CSF	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <
IL-13	↗	↗	←→	↗	↗	←→
CXCL10	OOR <	OOR <	OOR <	OOR <	OOR <	OOR <

FM, free mitochondria; MTD, mitochondrial lysate; mtDNA, mitochondrial DNA; OOR <, No signal detected in control and treatment groups; ↓, Signal detected in control, but no signal in treatment; ←→, No significant changes; ↗, Mediator elevated by treatments; ↘, Mediator suppressed by treatments.

Figure 23. FM induce cytokine and chemokine production in primary human CBMC at 6h and 24h co-culture.

CBMC were co-cultured with different concentration of FM, from 0 (Mock) to 400 μg/ml. FM400 μg/ml, equal to the mitochondria released by a 10% liver injury. (A) CCL2, (B) CCL3, (C) CCL4, (D) MMP9, (E) CXCL1, (F) CXCL8, (G) IL-6, (H) VEGF-A, (I) IL-1ra, (J) IL-1β, (K) IL-13, (L) IFNγ. FM induce pro-inflammatory mediator production at low doses in the CBMC (A-H), they also induce anti-inflammatory cytokine production at high doses in CBMC (I and K). FM do not induce IL-1β (J) and IFNγ (L) production in the CBMC supernatant. n=3 per group. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$.

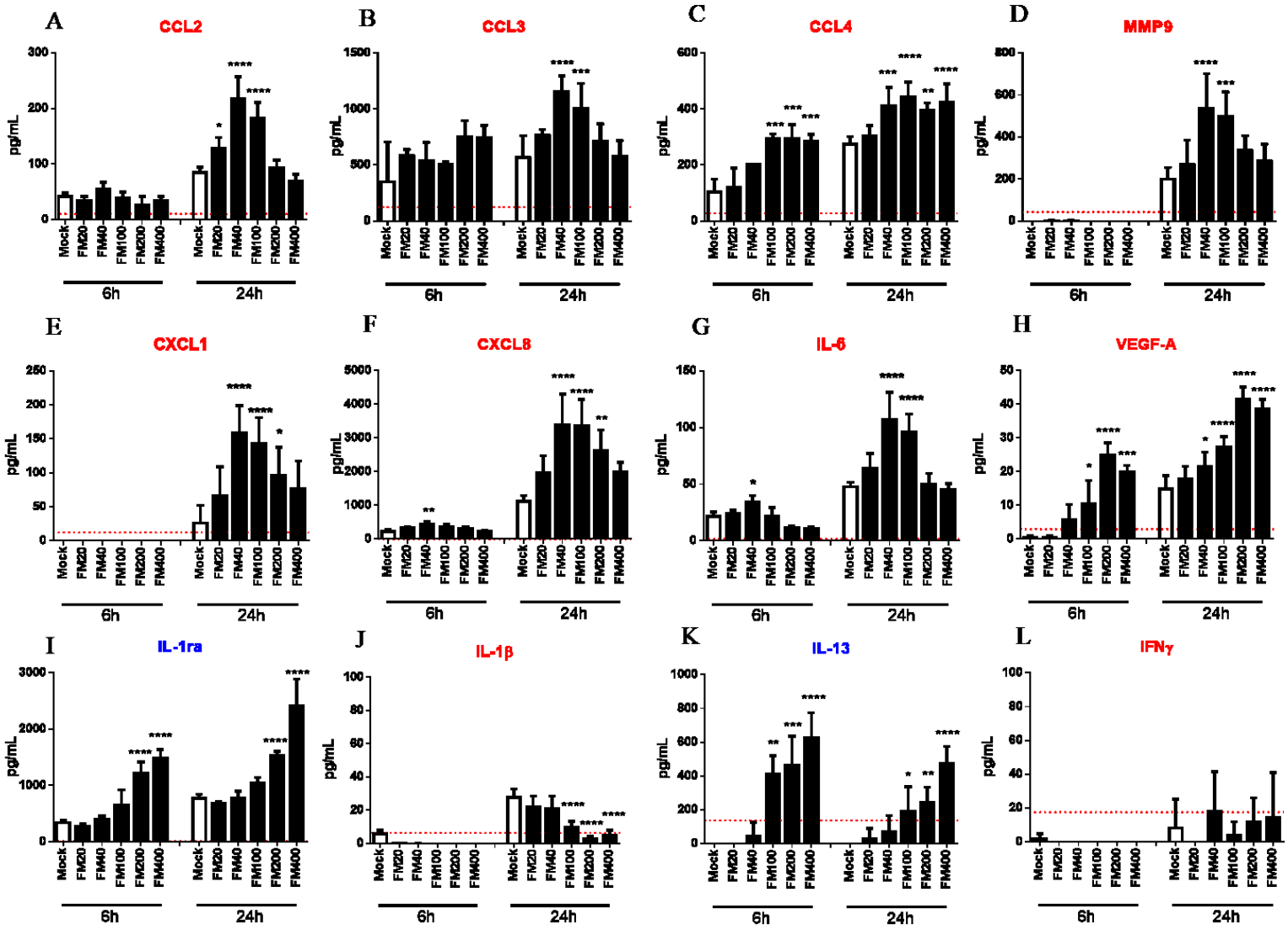

A
CCL2
B
CCL3
C
CCL4
D
MMP9
E
CXCL1
F
CXCL8
G
IL-6
H
VEGF-A
I
IL-1ra
J
IL-1β
K
IL-13
L
IFNγ
pg/mL
Mock
FM20
FM40
FM100
FM200
FM400
6h
24h

Figure 23

Figure 24. MTD induce inflammatory mediator production.

Mitochondrial lysate protein (MTD) induced anti-inflammatory cytokine production at high doses in primary human cord blood-derived mast cells (CBMC) at 6h and 24h co-culture but not pro-inflammatory mediators. CBMC were co-culture with different concentration of MTD, from 0 (Mock) to 400 μg/ml. MTD100 μg/ml, equal to the mitochondria released by a 10% liver injury. (A) CCL2, (B) CCL3, (C) CCL4, (D) MMP9, (E) CXCL1, (F) CXCL8, (G) IL-6, (H) VEGF-A, (I) IL-1ra, (J) IL-1β, (K) IL-13, (L) IFNγ. MTD induce anti-inflammatory cytokine (I and K) and VEGF-A (H) production at high doses , whereas not pro-inflammatory mediators (A-G) in the CBMC supernatant. n=3 per group. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

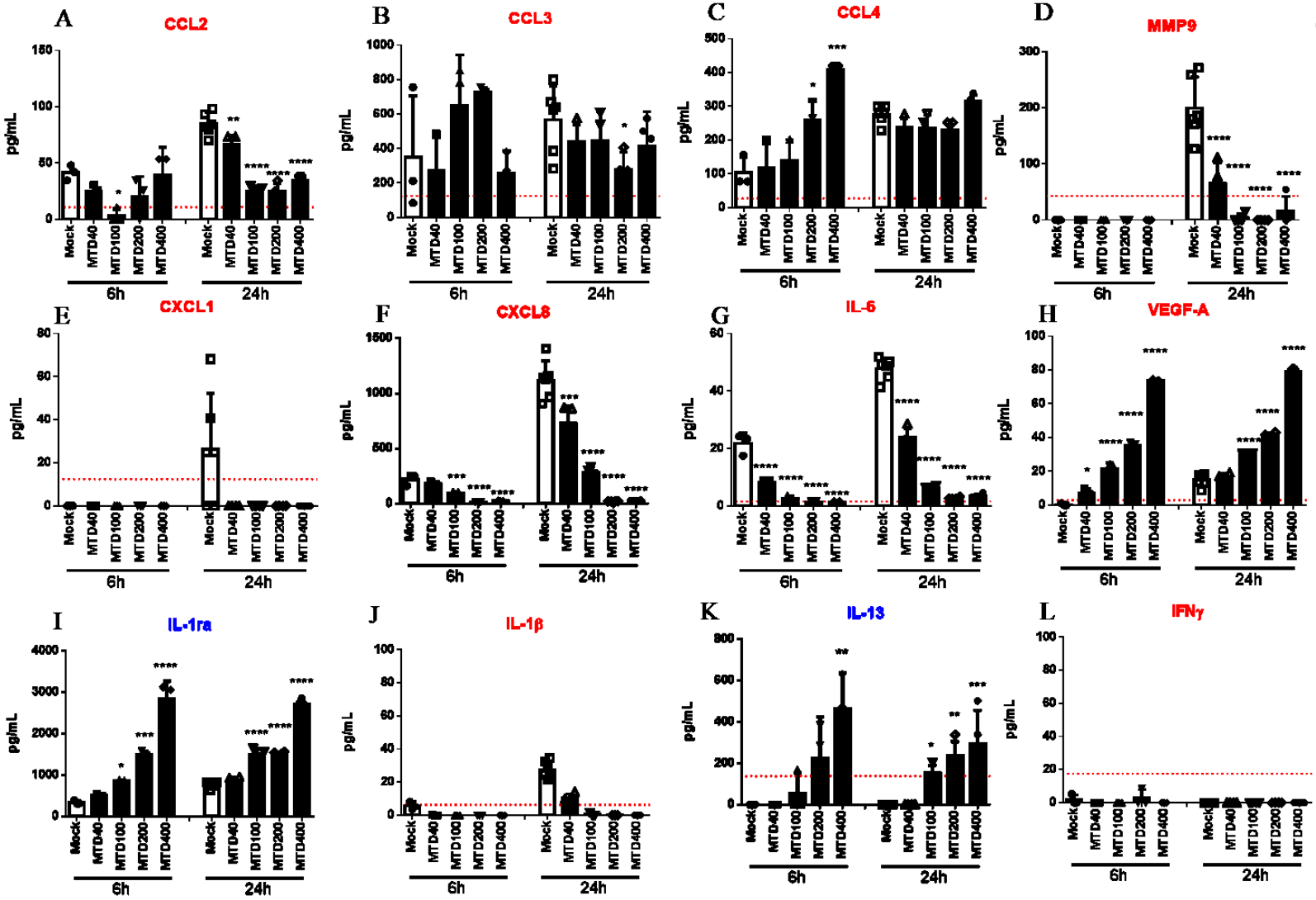

A
CCL2
B
CCL3
C
CCL4
D
MMP9
E
CXCL1
F
CXCL8
G
IL-6
H
VEGF-A
I
IL-1ra
J
IL-1β
K
IL-13
L
IFNγ
pg/mL
Mock
MTD40
MTD100
MTD200
MTD400
6h
24h

Figure 24

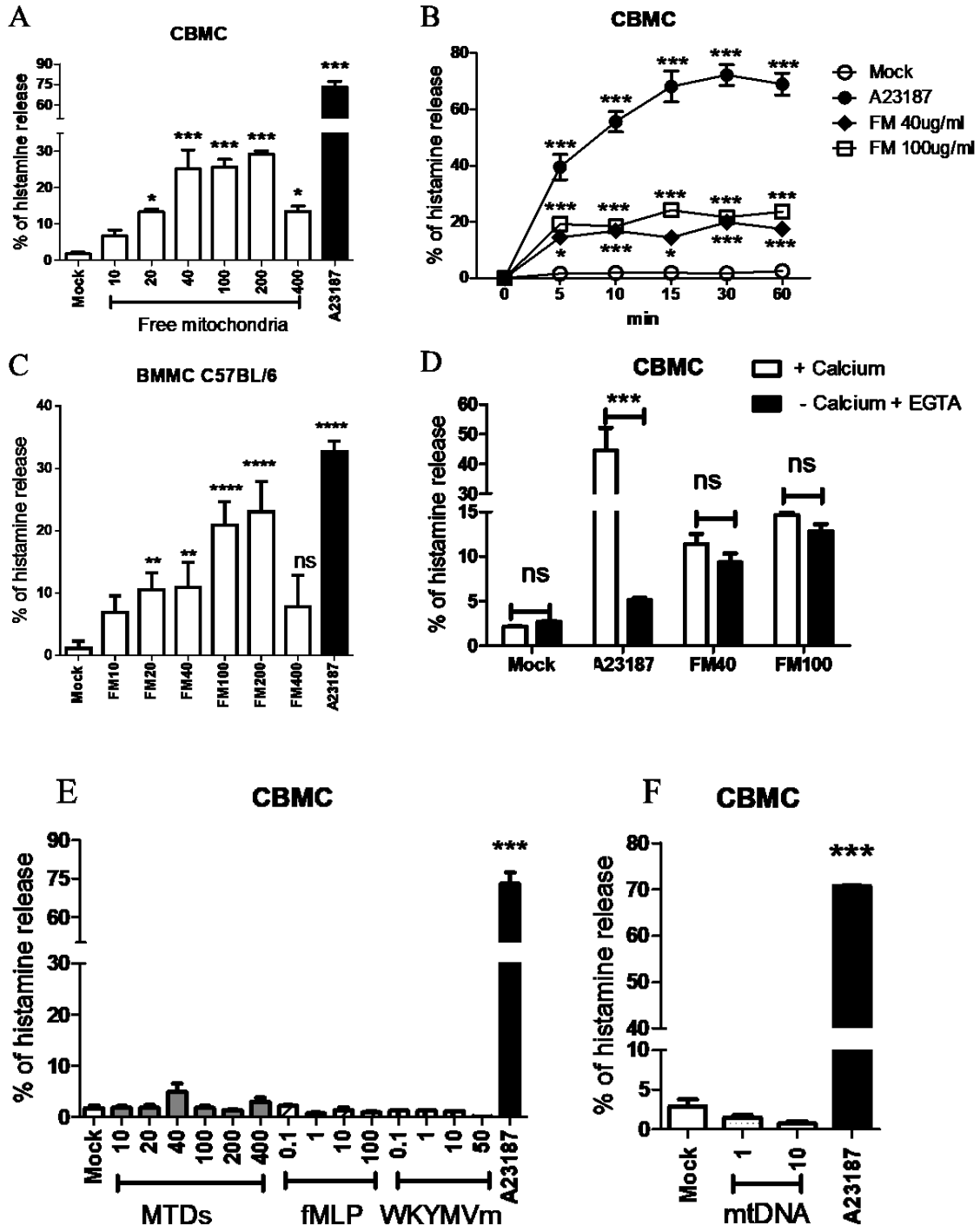
MC are filled with an abundance of secretory granules (SGs) where multiple mediators are stored (such as histamine, heparin, tryptases, chymase, carboxypeptidase A3, TNF, etc.) [263]. The process of preformed granule release, named degranulation, is tightly controlled and allows MC to rapidly and efficiently release bioactive mediators in response to extracellular stimuli [266]. The ability of FM, MTDs and mtDNA to induce MC degranulation was determined. Both human CBMC and murine bone marrow-derived cells (BMMC) were stimulated with a range of concentrations of homologous FM for 30min and degranulation was determined by examining histamine release. A23187 was used as a positive control. As shown in Figure 25, FM (20µg/ml to 200µg/ml) activated CBMC and BMMC to degranulate but MTDs and mtDNA did not share this effect even at relatively high concentrations (Figure 25E and F). Time course analysis showed that maximum degranulation, in response to FM, occurred within 15 min of stimulation of human MC with FM and induced approximately 20% degranulation by 5 min (Figure 25C).

MC degranulation is efficiently triggered by two main classes of receptors, FcRs and GPCR [266]. These mechanisms can differ in their requirements for extracellular calcium [266]. Calcium-free media containing a calcium chelator was used to investigate if FM-mediated MC degranulation required a calcium signal. As expected, calcium ionophore A23187 induced degranulation Figure 25D, and was significantly inhibited in the Ca^{2+} free (plus EGTA) media. However, FM could induce MC degranulation in both Ca^{2+} free and Ca^{2+} containing media (Figure 25D).

Figure 25. FM, but not mitochondrial protein or mtDNA can induce human primary CBMC and mouse BMMC degranulation.

The degranulation process induced by FM is rapid and largely calcium independent. (A), Human FM can induce human primary CBMC degranulation, n=6. (B), Mouse FM can induce BMMC degranulation, n=4. (C), FM induced histamine release is rapid, n=6. (D), this process is largely independent of extracellular calcium, n=6. FM, FM. A23187, Calcium ionophore A23187. MTDs, mitochondrial lysate protein. fMLP (FPR1 agonist), WKYMVm (agonist for the formyl peptide receptors FPR1, FPR2 and FPR3). *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 25


A
CBMC
% of histamine release
Mock
10
20
40
100
200
400
A23187
Free mitochondria
B
CBMC
% of histamine release
min
Mock
A23187
FM 40ug/ml
FM 100ug/ml
C
BMMC C57BL/6
% of histamine release
Mock
FM10
FM20
FM40
FM100
FM200
FM400
A23187
D
CBMC
+ Calcium
- Calcium + EGTA
% of histamine release
ns
Mock
A23187
FM40
FM100
E
CBMC
% of histamine release
Mock
MTDs
fMLP
WKYMVm
A23187
F
CBMC
% of histamine release
Mock
mtDNA
A23187

3.2.5. Effect of inhibition FPR1 in MC activation *in vitro*

The potential receptors mediating MC activation in response to FM were considered. Previous literature has reported that mitochondrial DAMPs including ATP, mtDNA (unmethylated CpG motifs), and formyl peptides, may induce cellular activation through distinct receptor systems (e.g., P_2X_7, Toll-like receptor and FPR1, respectively) [69, 318, 319]. Formyl peptide receptors (FPRs) belong to a family of GPCRs, which recognize bacterial and mitochondrial formylated peptides as well as endogenous non-formylated peptides. We examined FPR expression by MC in the context of FM stimulation. *In vitro* cultured CBMC showed mRNA expression for FPR1, FPR2 and FPR3 by regular PCR (Figure 26A). The mRNA expression level of FPR1 was significantly increased in response to FM (Figure 26B), the expression levels of FPR2 and FPR3 remained below the level of detection by qPCR. Protein expression of FPR1 on the CBMC was confirmed by flow cytometry (Figure 26C).

To determine the function of FPRs in MC responses to FM, we utilized pharmacological inhibitors known to block FPR1 function (CsH and Boc-1) or FPR2 (WRW4). When CBMC were treated with CsH or Boc-1, the CCL3 and CXCL8 responses to FM were significantly reduced by FPR1 inhibitor treatment, while the FPR2 antagonist did not show a significant inhibitory effect (Figure 26D and E). FM also induced BMMC activation and production of IL-6 (Figure 26F), a response that was significantly decreased by FPR1 inhibitor treatment (Figure 26G). Pretreatment with FPR1 inhibitors also resulted in a decrease in histamine release (Figure 26H and I) in response to FM. In contrast, responses of A23187-stimulated BMMC were not significantly modified by FPR inhibitors (Figure 26H and I). We also investigated other potential receptors via the use of alternate

ligands. mtDNA did not induce MC activation (Figure 25 and Table 4). ATP also did not induce CBMC activation (data not shown) using standard readouts. A P_2X_7 inhibitor, anti-human TLR2 and TLR4 antibodies were also used in CBMC degranulation responses to FM, but none of them attenuated degranulation (data not shown).

Figure 26. FPR1-dependent signals in FM induced MC activation and degranulation. (A) Expression of FPRs mRNA in human primary CBMC. (B) The expression level of FPR1 was elevated by FM 100μg/ml, whereas the ct value for FPR2 and FPR3 was larger than 35. (C) Expression of FPR1 protein in human primary CBMC. The FPR1 inhibitors (Boc-1 5uM and Cyclosporin H, CsH 1μM) can inhibit CBMC activation induced by FM whereas FPR2 antagonist (WRW_4, 5μM) does not have the inhibitory function. The production of CCL3 (D) and CXCL8 (E) at 24h of FM and CBMC coculture. (F) Mouse FM induce BMMC to secrete IL-6 into the supernatant at 24h co-culture. (G) FPR1 inhibitors (Boc-MLF 5μM and CsH 1μM) inhibit IL-6 production in the BMMC. (H-I) The inhibitory role of FPR1 inhibitors (CsH and Boc-MLF) was confirmed in the reduced BMMC degranulation induced by FM. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

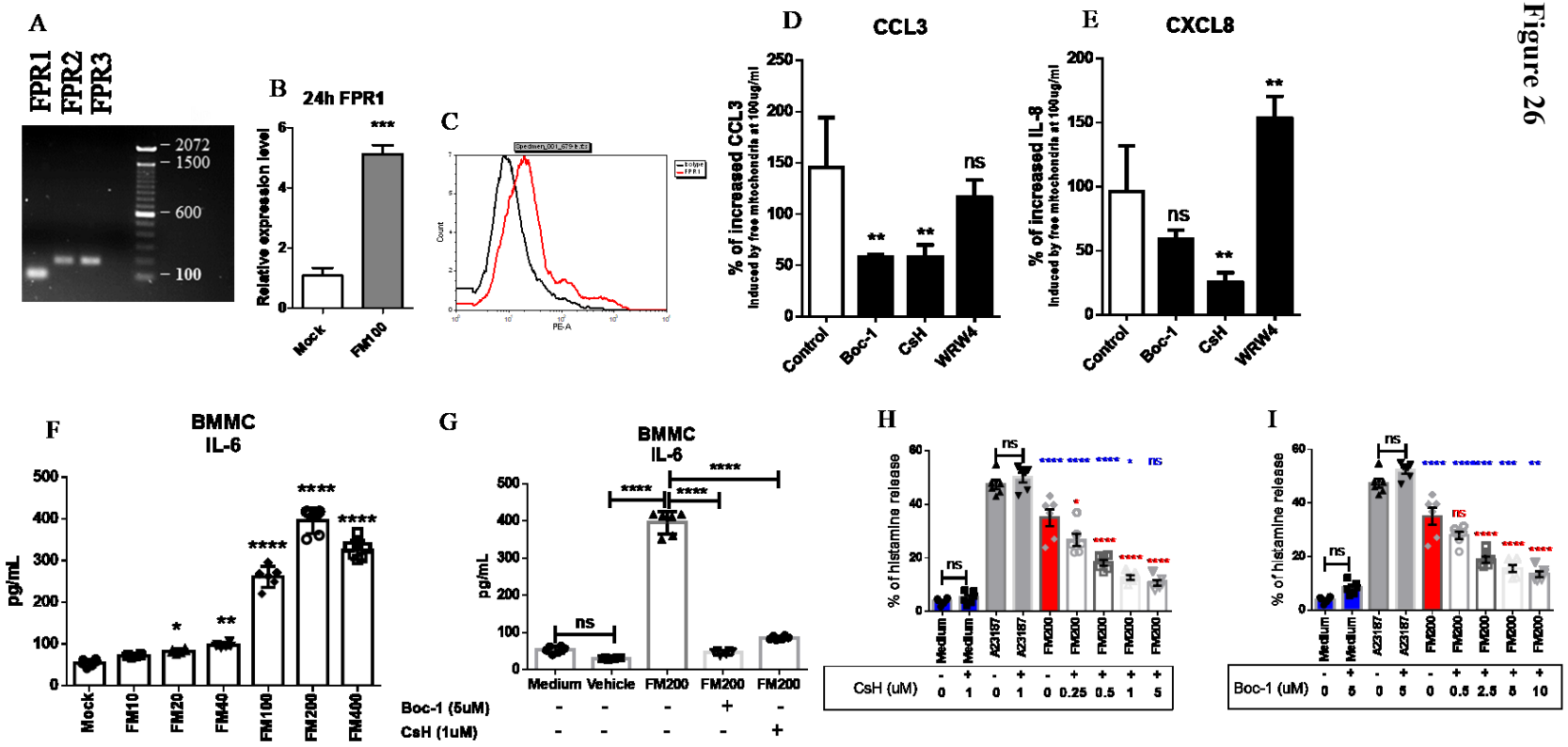

A
FPR1
FPR2
FPR3
2072
1500
600
100
B
24h FPR1
Relative expression level
Mock
FM100
C
PE-A
D
CCL3
% of increased CCL3
Induced by free mitochondria at 100ug/ml
Control
Boc-1
CsH
WRW4
E
CXCL8
% of increased IL-8
Induced by free mitochondria at 100ug/ml
F
BMMC
IL-6
pg/mL
Mock
FM10
FM20
FM40
FM100
FM200
FM400
G
BMMC
IL-6
pg/mL
Medium
Vehicle
FM200
Boc-1 (5uM)
CsH (1uM)
H
% of histamine release
Medium
A23187
FM200
CsH (uM)
I
% of histamine release
Boc-1 (uM)

Figure 26

3.2.6. FPR1 inhibition prevents peritonitis triggered by FM *in vivo*

The ability of FPR1 inhibitors to modify the influx of leukocytes in response to FM was examined *in vivo*. tSNE plots show the distribution of immune lineages from mice pre-injected with CsH for 30min, the i.p injections of FM (Figure 27A) compared with those only injected with PBS or FM. We found that the FPR1 inhibitor CsH prevented peritonitis induced by FM, as indicated by significantly reduced total peritoneal cell responses as well as reduced influx of specific populations of infiltrating leukocytes, such as neutrophils ($CD11b^+$ $Ly6C^+Ly6G^+$), inflammatory macrophages ($CD11b^+Ly6C^+Ly6G^-$), DC ($CD11c^+MHCII^+$), pDC ($CD11c^+MHCII^+CD317^+B220^+$), TDC ($CD11c^+MHCII^+CD103^+$), MC ($CD117^+Fc\varepsilon RI^+$), and eosinophils (Siglec-$F^+CD11b^{-/lo}$), from the FM injected group versus FM pre-treated with CsH (Figure 27B-J). Control groups, given PBS with or without CsH pre-treatment, had no significant background changes (Figure 27B-P). Interestingly, we found that the increased number and activation of MC was completely eliminated by FPR1 inhibition (Figure 27K), while the responses of other leukocytes, such as neutrophils, were substantially attenuated (Figure 27 C, E, I and L). There was no significant reduction of NK cell infiltration when mice were pretreated with CsH (Figure 27L). The resting macrophages shift was not observed either (Figure 27A and D). Notably, most lymphocyte lineages stayed in normal range after FM injection regardless of CsH pretreatment (Figure 27M-P). Control experiments using FSL-1 (TLR2 activator) injected mice confirmed that CsH inhibition was selective in reducing responses to FPR1 but not to the TLR2 ligand (Figure 28). The percentage differences of neutrophils and macrophages in these and related experiments are shown in Figure 29. Collectively, these data indicate that inhibition of FPR1 with CsH can prevent myeloid cell infiltration and MC activation by FM *in vivo*.

Figure 27. The role of FPR1 in FM induced peritoneal inflammation.

C57BL/6 mice were injected i.p. with PBS or FM (5mg/kg) (equal to the mitochondria released by a 2.5% liver injury), or pre-injected with FPR1 inhibitor, Cyclosporin H (2mg/kg) 30 min before FM injection. After 16 hour the peritoneal contents were harvested by lavage, (A) the total peritoneal cavity cells (PCC) were counted, and the percentage of leukocytes in each sample were identified by flow cytometry. (B)Neutrophils, (C) Resting macrophages, (D) Inflammatory macrophages, (E) Dendritic cells, (F) Plasmacytoid dendritic cells (pDC), (G) Tolerogenic dendritic cells (TDC), (H) Eosinophils, (I) MC, (J) $CD69^+$ MC, (K) Natural killer cells (NK), (L) $CD3^+$ T cells, (M) $CD4^+$ T helper cells, (N) CD8+ Cytotoxic T cells, (O) B cells. (O) and (P) Staining represents the phenotype of myeloid cells for both PBS and FM injected C57BL/6 mouse. The graphs shown are the mean±SEM, PBS group n=11; FM group n=10. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 27

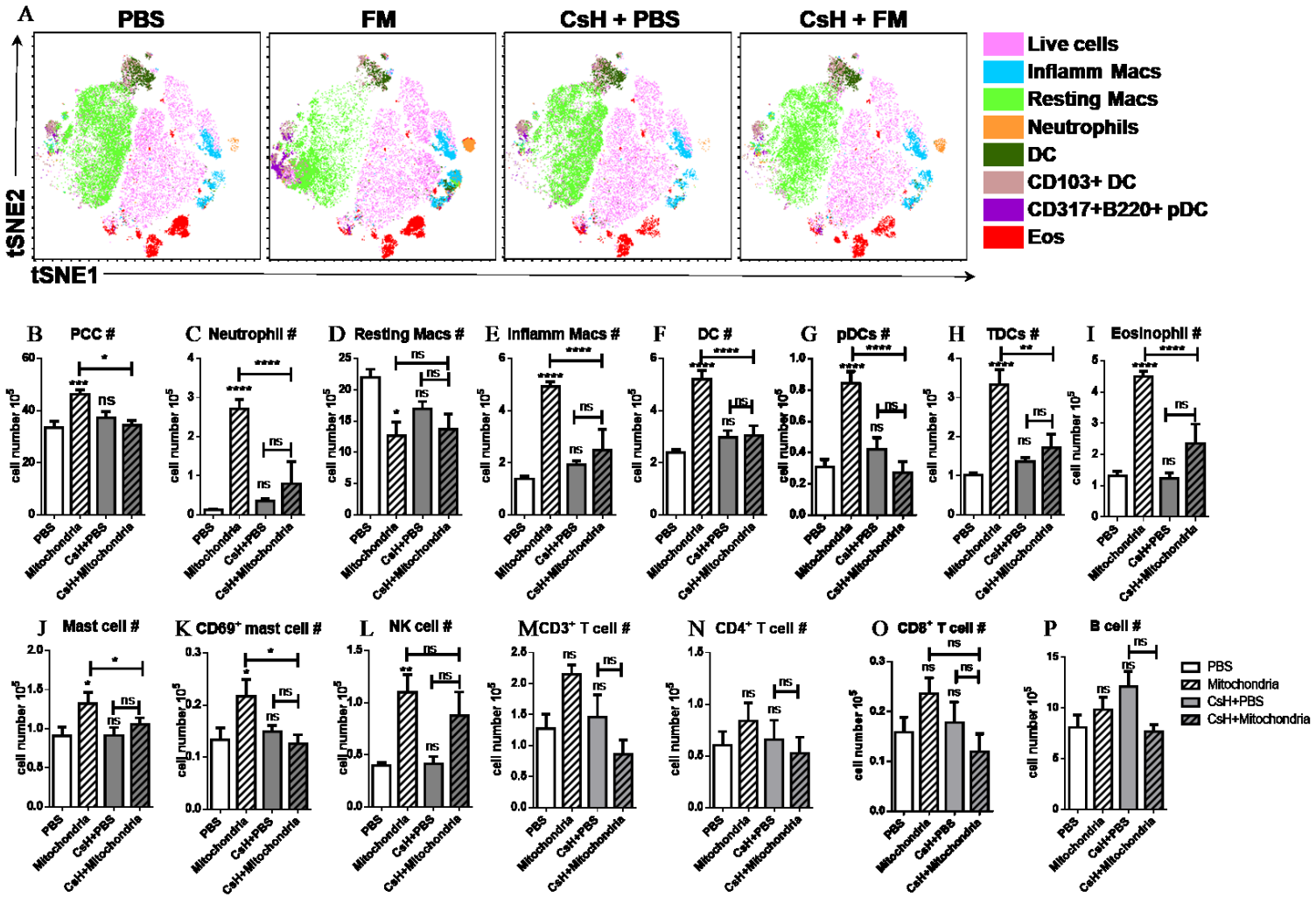

A
PBS
FM
CsH + PBS
CsH + FM
tSNE2
tSNE1
Live cells
Inflamm Macs
Resting Macs
Neutrophils
DC
CD103+ DC
CD317+B220+ pDC
Eos
B PCC #
C Neutrophil #
D Resting Macs #
E Inflamm Macs #
F DC #
G pDCs #
H TDCs #
I Eosinophil #
J Mast cell #
K CD69+ mast cell #
L NK cell #
M CD3+ T cell #
N CD4+ T cell #
O CD8+ T cell #
P B cell #
cell number 10^5
PBS
Mitochondria
CsH+PBS
CsH+Mitochondria
ns
*
**
***
****

Figure 28. The FPR1 inhibitor CsH can not reduce peritoneal inflammation induced by TLR2 ligand FSL-1.

C57BL/6 mice were injected i.p. with PBS or FSL-1 (Pam2CGDPKHPKSF) (1μg per mouse), or pre-injected with Cyclosporin H (2mg/kg) 30 min before FSL-1 injection. After 16 hours the peritoneal contents were harvested by lavage, (A) total peritoneal cavity cells (PCC) were counted, and the percentage of leukocytes in each sample was identified by flow cytometric analysis. (B)Neutrophils, (C) Resting macrophages, (D) Inflammatory macrophages, (E) Dendritic cells, (F) Plasmacytoid dendritic cells (pDC), (G) Tolerogenic dendritic cells (TDC), (H) Eosinophils, (I) MC, (J) $CD69^{+}$ MC, (K) Natural killer cells (NK). The graphs shown are the mean±SEM, PBS group n=11; FSL-1 group n=5; CsH+PBS group n=8; CsH+FSL-1 group n=5. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$.

Figure 28

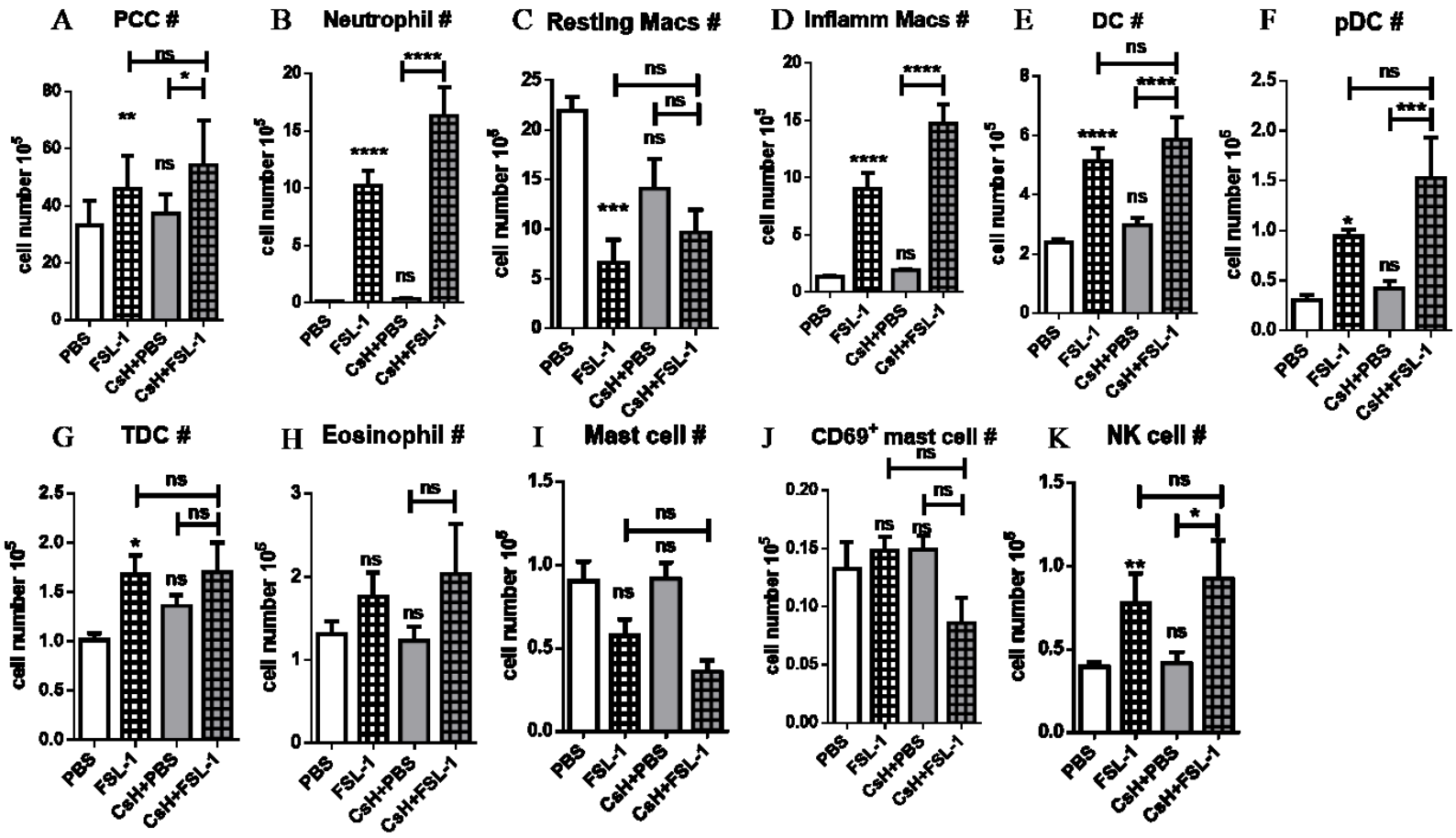
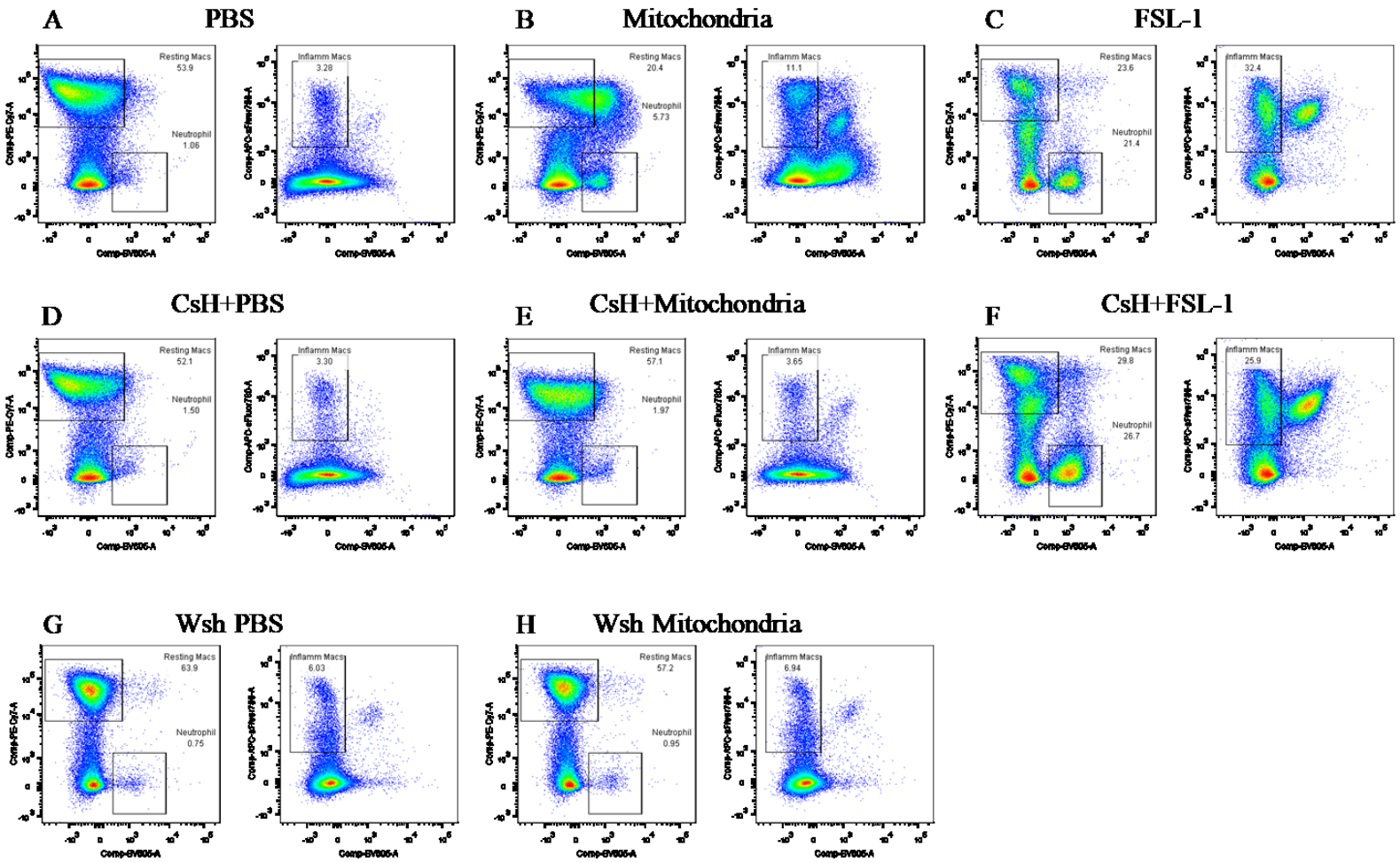

A PCC #
B Neutrophil #
C Resting Macs #
D Inflamm Macs #
E DC #
F pDC #
G TDC #
H Eosinophil #
I Mast cell #
J CD69+ mast cell #
K NK cell #
cell number 10^5
PBS
FSL-1
CsH+PBS
CsH+FSL-1
ns
*
**
***
****

Figure 29. The phenotype of myeloid cells in mouse peritoneum changes with different injection contents and different mouse strains.

Staining is shown for a PBS, FM (5mg/kg), FSL-1 (1µg per mouse) injected C57BL/6 mouse or PBS and FM (5mg/kg) injected C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) MC deficient mouse at 16h. After an initial FSC/SSC scatter gate, a sequential gating strategy was used to identify populations expressing specific markers: neutrophils (CD11b^{+} F4/80^{-}Ly6G^{+}), resting macrophages (resting Macs, CD11b^{+}F4/80^{hi}Ly6G^{-}), inflammatory macrophages (Inflamm macs, CD11b^{+}Ly6C^{+}Ly6G^{-}). FSC, forward scatter; MHCII, major histocompatibility complex class II; SSC, side scatter.

Figure 29


A
PBS
Resting Macs
53.9
Neutrophil
1.06
Inflamm Macs
3.28
B
Mitochondria
Resting Macs
20.4
Neutrophil
5.73
Inflamm Macs
11.1
C
FSL-1
Resting Macs
23.6
Neutrophil
21.4
Inflamm Macs
32.4
D
CsH+PBS
Resting Macs
52.1
Neutrophil
1.50
Inflamm Macs
3.30
E
CsH+Mitochondria
Resting Macs
57.1
Neutrophil
1.97
Inflamm Macs
3.65
F
CsH+FSL-1
Resting Macs
29.8
Neutrophil
26.7
Inflamm Macs
25.9
G
Wsh PBS
Resting Macs
63.9
Neutrophil
0.75
Inflamm Macs
6.03
H
Wsh Mitochondria
Resting Macs
57.2
Neutrophil
0.95
Inflamm Macs
6.94

3.3 Extracellular mitochondria induce macrophage activation

Given the role of extracellular mitochondrial components in hepatocyte survival rate and immune sentinel cells (MC) activation, whether liver IR could affect the most abundant liver immune cells (macrophages) was questioned. It was also important to determine if extracellular mitochondrial components could activate macrophages. Apart from parenchymal cells, KC are known as tissue-resident macrophages and account for 20-35% of all non-parenchymal cells in the liver and 80-90% of tissue macrophages present in the body [320]. Similar to other macrophages, KC also sense endogenous molecular signals that may result from perturbed homeostasis of the host. In this section, how the macrophages react to liver IR and respond to extracellular mitochondria are explored.

3.3.1 IR induces different levels of mitochondrial damages *in vitro* depending on macrophage phenotypes

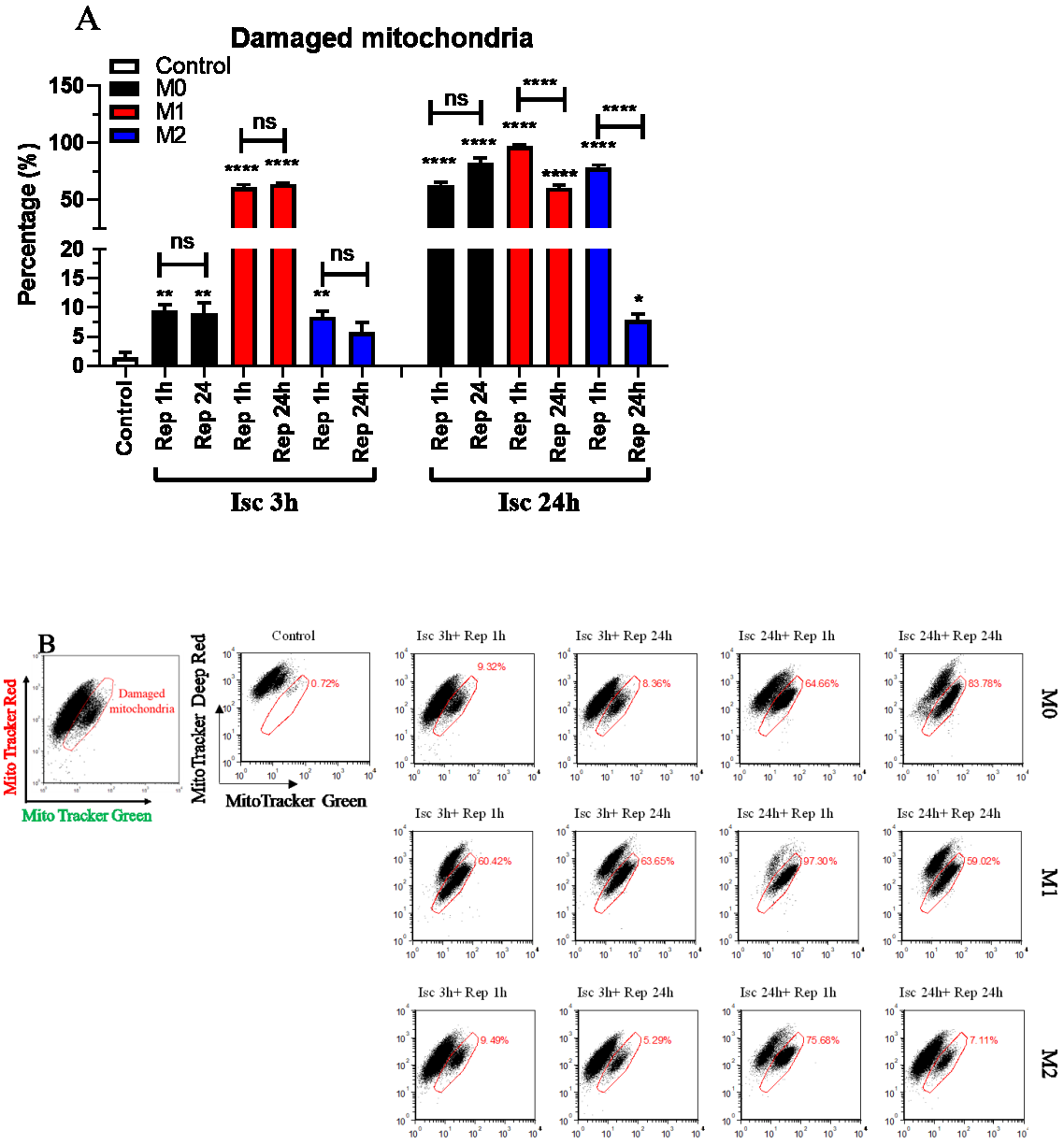
The functional plasticity of macrophages is driven by their immunological environment that can shape their properties through a wide spectrum of phenotypes, among which classical (M1) or alternative (M2) represent the extreme states. Given that macrophages have different phenotypes in tissue, the ability of IRI to induce similar levels of mitochondrial damage in different phenotypes of macrophages was determined. To this end, a mitochondrial damage assay was performed by staining cells with MitoTracker Deep Red and MitoTracker Green. Three types of macrophages (M0, M1, M2) were stressed to two ischemia timepoints (3h and 24h) and before returning to normal conditions for 1h or 24h to mimic different timepoints of IR *in vitro*. A shifted subpopulation to MitoTracker Green positive indicated cells with mitochondrial damage. M1 macrophages were found to be most susceptible to such IR stress, they had more than 60% cells containing damaged

mitochondria at 3h ischemia plus 1h reperfusion condition (IR 3h+1h) and 97% cells containing damaged mitochondria at IR 24h+1h; M1 macrophages were observed to have a recovery ability (percentage of mitochondrial damage decreased to 60% from 97%) when they suffered severe stress, comparing conditions between IR24h+1h and IR24h+24h (Figure 30). Similar to M1 macrophages, both M0 and M2 macrophages also suffered mitochondrial damage in response to IR stress *in vitro*, however, M0 and M2 macrophages also displayed different recovery abilities. In Figure 30, when M0 underwent a severe ischemic stress (24h ischemia), the mitochondrial damage worsened after 24h of reperfusion compared to a transient reperfusion (1h). Interestingly for M2 macrophages, although severe ischemic stress (24h ischemia) induced huge mitochondrial damage, after 24h of reperfusion, the percentage of cells containing damaged mitochondria dropped dramatically from 78% to 7% (Figure 30). Together, the recovery ability of different phenotypes of macrophages suggested that M0 and M1 might be more susceptible to hepatic IRI.

Figure 30. IR *in vitro* induces different levels of mitochondrial damages in M0, M1 and M2 macrophages.

Mouse BMDM were stressed for 3h or 24h ischemia, followed by re-oxygenation for 1h or 24h. Mitochondrial damage was determined by MitoTracker Deep Red and MitoTracker Green staining. (A) Percentage of damaged mitochondria is indicated by MitoTracker Green positive cells. (B) M0, M1 and M2 were stained with MitoTracker Deep Red and MitoTracker Green, representative plots are shown. All values are means ± SD of 3 independent experiments. *, $P< 0.05$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 30


A
Damaged mitochondria
Control
M0
M1
M2
Percentage (%)
150
100
50
20
15
10
5
0
ns
****
**
*
Control
Rep 1h
Rep 24
Rep 1h
Rep 24h
Rep 1h
Rep 24h
Rep 1h
Rep 24
Rep 1h
Rep 24h
Rep 1h
Rep 24h
Isc 3h
Isc 24h
B
Mito Tracker Red
Mito Tracker Green
Damaged mitochondria
Control
0.72%
MitoTracker Deep Red
MitoTracker Green
Isc 3h+ Rep 1h
Isc 3h+ Rep 24h
Isc 24h+ Rep 1h
Isc 24h+ Rep 24h
9.32%
8.36%
64.66%
83.78%
60.42%
63.65%
97.30%
59.02%
9.49%
5.29%
75.68%
7.11%
M0
M1
M2

3.3.2 Mitochondrial components induce macrophage death *in vitro*

Having demonstrated a role for extracellular mitochondrial components in inducing hepatocyte death (Figure 5) whether macrophages as the most abundant immune cells in liver would be similarly affected was evaluated. As shown in Figure 30, M0 and M1 macrophages were more susceptible to IRI. Whether M0 and M1 macrophages survival rates would be affected by extracellular mitochondrial components which were demonstrated to be released in liver IRI was also investigated. FM, MTDs and mtDNA were used to co-culture with M0 and M1 macrophages respectively, no significant cell death was observed by co-culturing with FM or mtDNA (data not shown), however, MTDs induced significant M0 and M1 macrophage cell death at concentrations of 200μg/ml and 400μg/ml (Figure 31). Moreover, M1 macrophages (68% survival rate at MTD400, Figure 31 B) were more susceptible to MTDs compared to M0 (75% survival rate at MTD400, Figure 31A).

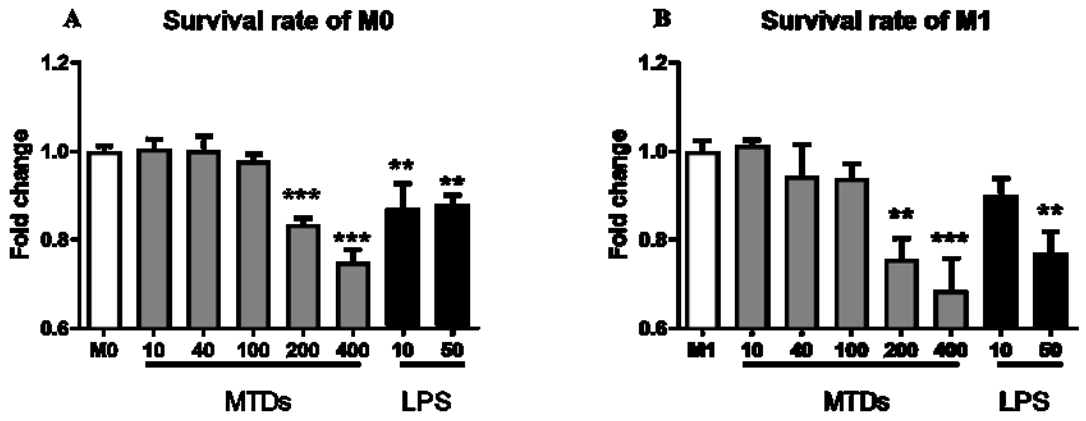


Figure 31. MTDs cause M0 and M1 macrophage death *in vitro*.

A, MTDs (Mitochondrial lysate) induce significant M0 mouse macrophage cell death at concentrations above 200 μg/ml, RAW264.7 mouse macrophage cell was line co-cultured with different concentrations of MTDs (μg/mL) for 24 hours;

B, MTDs induce significant M1 mouse macrophage cell death at concentrations above 200 μg/ml. RAW264.7 mouse macrophage cell line was primed with 10ng/ml LPS to achieve a M1 phenotype, then washed and co-cultured with different concentrations of MTDs for 24 hours (μg/ml).

LPS, lipopolysaccharide was set as a positive control. *, P< 0.05; **, P<0.01; ***, P<0.0005; ****, P< 0.0001 (n=4/group).

3.3.3 FM enhance pro-inflammatory cytokine production in M1 macrophages independent of the FPR1 receptor

Given that extracellular mitochondrial components induced MC activation both *in vitro* and *in vivo*, I further assessed whether another important tissue resident immune cells in liver, macrophages, could similarly respond to mitochondrial components. First, whether mitochondrial components could induce macrophages polarization or not was evaluated. Mature BMDM of the M0 phenotype were co-cultured with FM (from doses of 10μg/ml to 400μg/ml), MTDs (from 10μg/ml to 400μg/ml) and mtDNA (from 1μg/ml to 20μg/ml), but none of these mitochondrial components induced M0 macrophages to polarize to M1 or M2 phenotypes (data not shown). Next, to determine whether extracellular mitochondria could induce macrophages to activate inflammatory responses indicated by cytokine production, M1 BMDM were used in co-culture with different mitochondrial components. IL-1β, TNF and IL-6 were measured as indicators of cytokine expression. At 24 hours post-stimulation, only FM induced significantly increase expression of TNF, IL-1β and IL-6 in M1 macrophages (Figure 32 A and C). Although mtDNA and MTDs also enhanced TNF expression, the elevated level of TNF in response to FM was much higher that the other mitochondrial stimuli (Figure 32 B). Next, TNF levels were measured as an indicator of cytokine production in culture supernatants by ELISA. Consistently, FM significantly enhanced TNF production in M1 macrophages Figure 33).

Having demonstrated that the production of pro-inflammatory responses in M1 macrophages could be significantly enhanced in response to extracellular FM, primary M1 macrophages were incubated with FM or MTDs or media alone for 24 hours, and supernatants were analyzed for the presence of 18 cytokines and chemokines through a

Luminex assay. In culture supernatants from primary mouse M1 macrophages stimulated with FM, elevated levels of IL-1β, IL-6, CCL3, MMP9, CXCL1, CCL2 and CCL5 were detected, compared to media controls (Figure 34). Conversely, supernatants from MTDs-stimulated M1 macrophages had concentrations of these mediators close to baseline (Figure 34). In addition, a mouse macrophage cell line was used to confirm how M1 macrophages respond to extracellular mitochondria. Although no enhancement of cytokine production was observed in primary M1 macrophages in response to MTDs, a significant increase of VEGF-A and reduction of MMP9 production were observed from the M1 macrophage cell line (Figure 35). Consistent with primary mouse M1 macrophages, FM induced significant elevations of IL-1β, IL-6, MMP9, CXCL1, VEGF-A, IL-13 and CCL5 production compared to control cells (Figure 35).

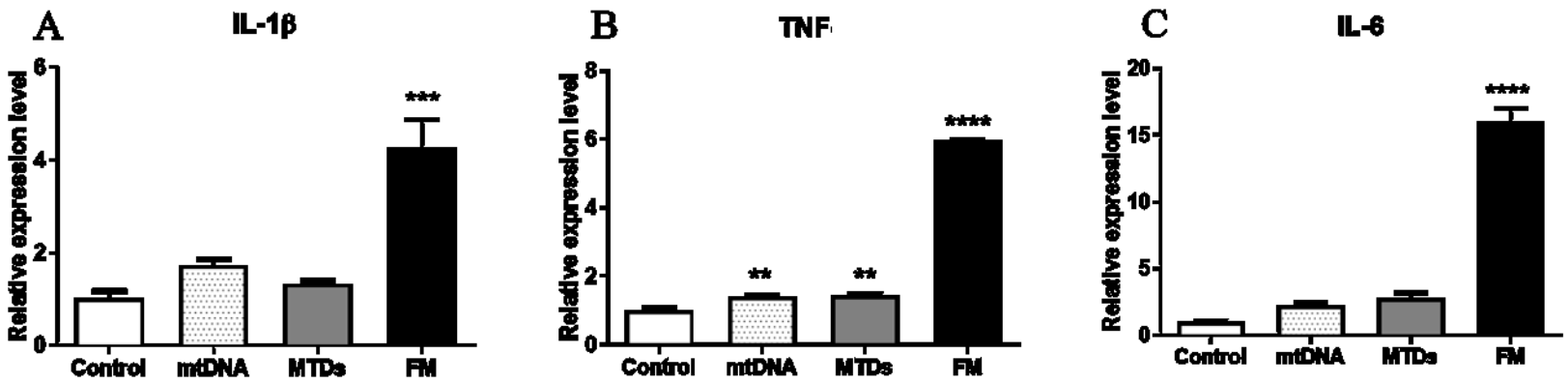


Figure 32. Mouse M1 macrophages elevate inflammatory cytokine expression in response to FM but not mtDNA or MTDs. Mouse bone marrow-derived macrophages were primed with IFNγ 10ng/ml for 24 hours, then co-cultured with mtDNA (10μg/ml), MTDs (200μg/ml) and FM (FM, 200μg/ml) for 24h. The relative expression levels were normalized to *GUSB* and *HPRT* housekeeping genes. Relative mRNA expression of (A) IL-1β, (B) TNF and (C) IL-6. The graphs shown are the mean±SEM, n=3. **, P<0.01; ****, P< 0.0001.

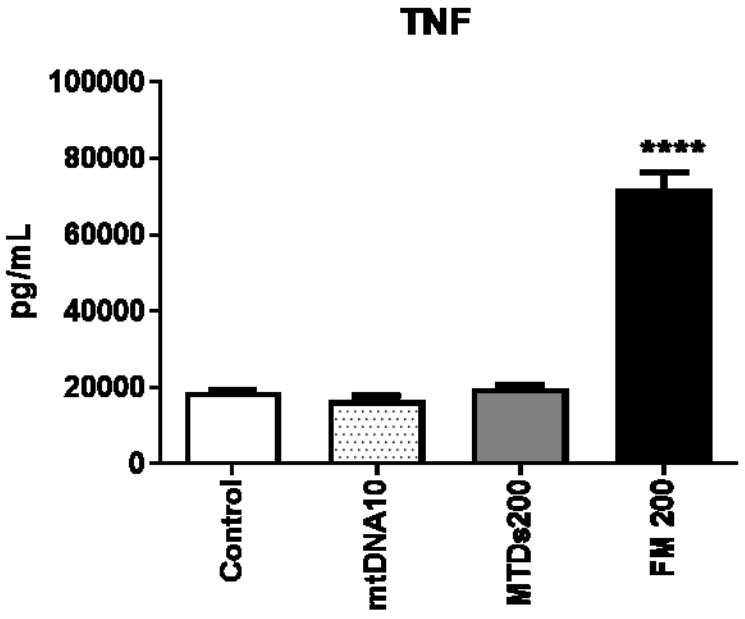


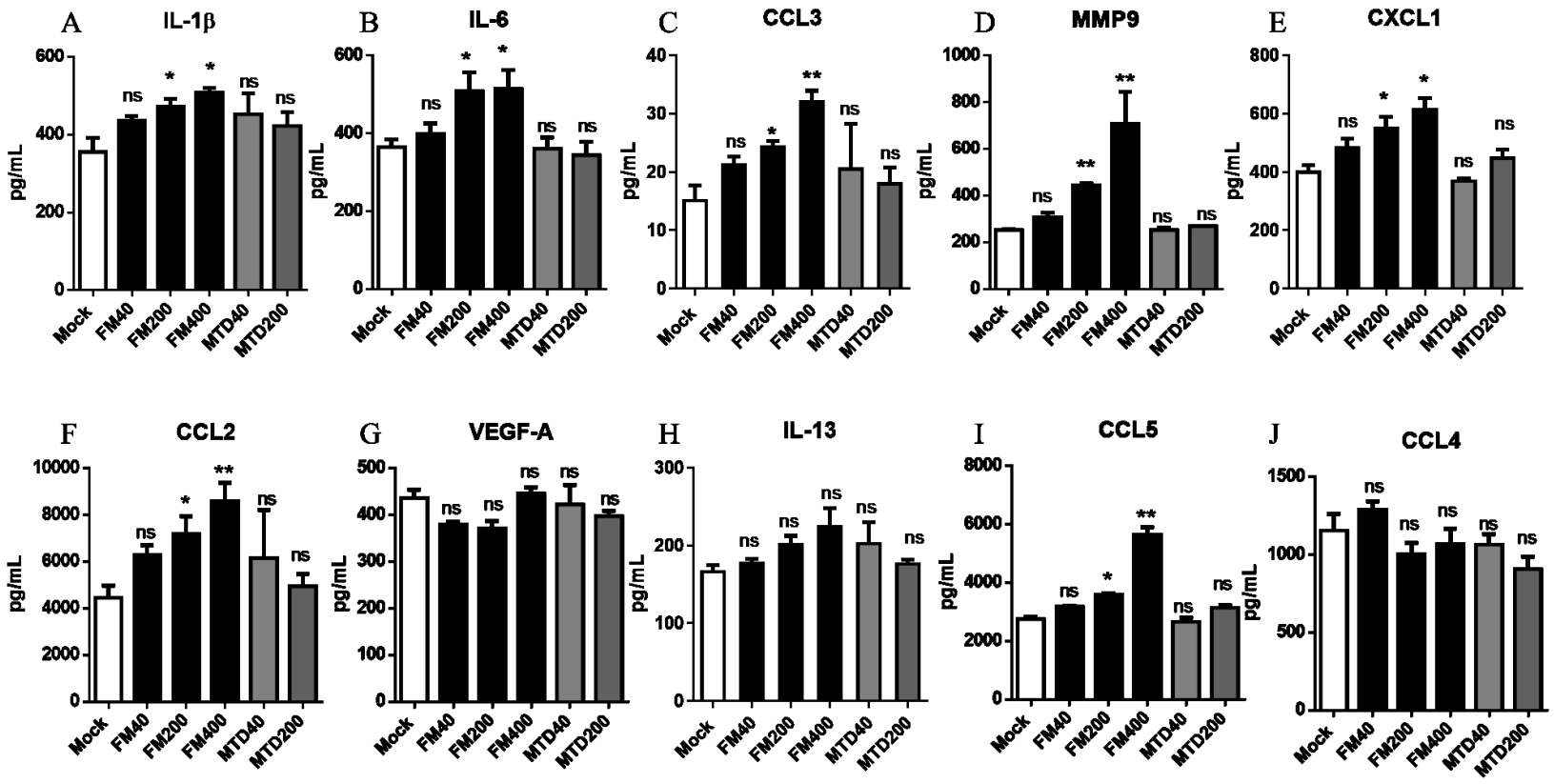
Figure 33. FM enhance TNF production in M1 macrophages.

Mouse RAW264.7 macrophage cell line was primed with LPS 10ng/ml for 4 hours, then co-cultured with mtDNA (10μg/ml), MTDs (200μg/ml) and free mitochondria (FM, 200μg/ml) for 24h. The production of TNF in supernatants were evaluated by ELISA. The graphs shown are the mean±SEM, n=4. ****, P< 0.0001.

Figure 34. FM induce cytokine and chemokine production in primary mouse bone marrow-derived macrophages after 24h co-culture.

BMDM were primed with 10ng/ml LPS for 3h, then co-cultured with different concentrations of free mitochondria (FM) from 0 (Mock) to 400 μg/ml or mitochondrial lysate (MTDs) from 0 to 200 μg/ml. FM400 μg/ml or MTDs 100μg/ml is equal to the mitochondria released by a 10% liver injury. (A) IL-1β, (B) IL-6, (C) CCL3, (D) MMP9, (E) CXCL1, (F) CCL2, (G) VEGF-A, (H) IL-13, (I) CCL5 and (J) CCL4. n=3 per group. *, $P< 0.05$; **, $P<0.01$.

Figure 34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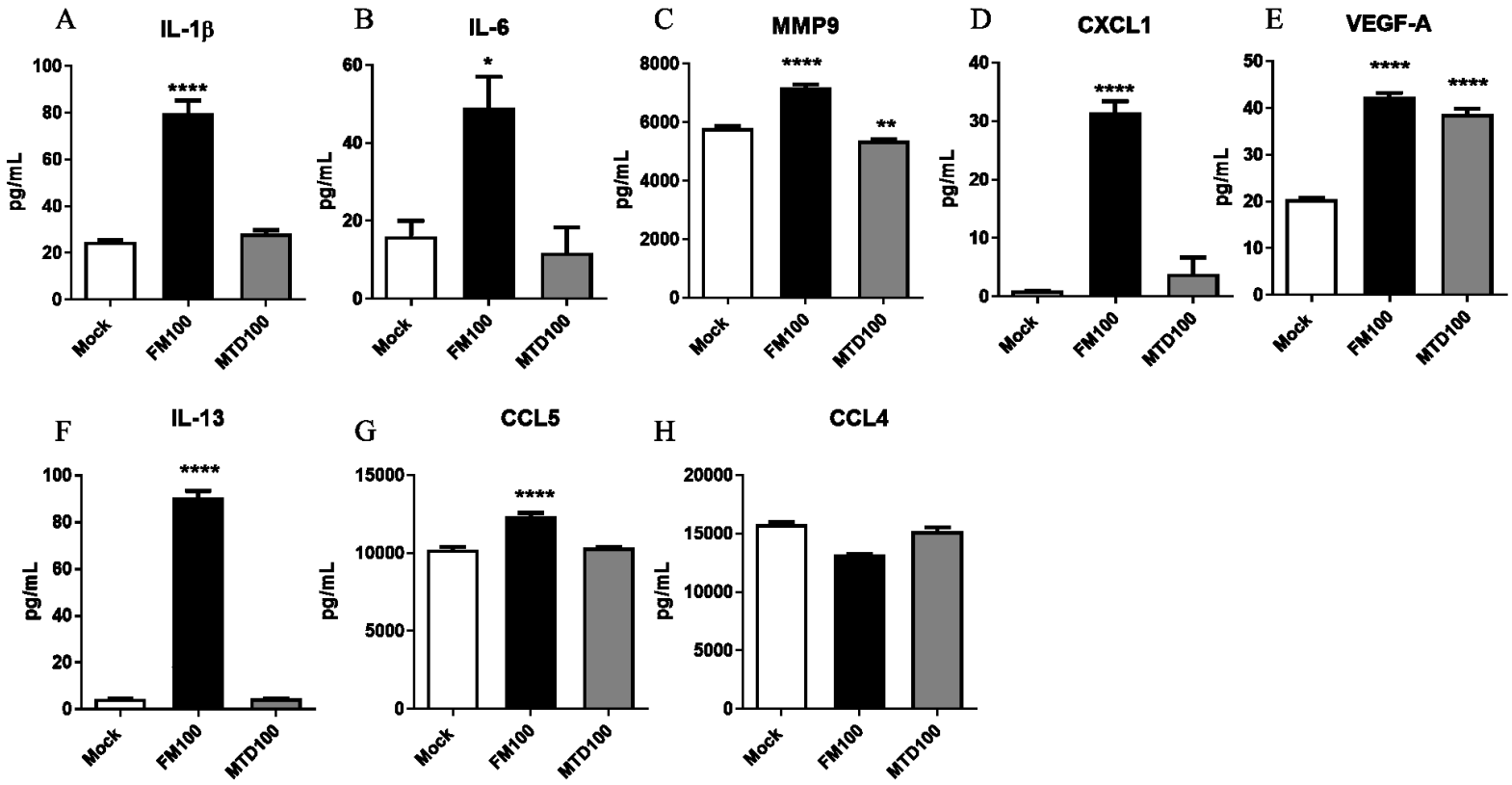
IL-1β
B
IL-6
C
CCL3
D
MMP9
E
CXCL1
F
CCL2
G
VEGF-A
H
IL-13
I
CCL5
J
CCL4
pg/mL
Mock
FM40
FM200
FM400
MTD40
MTD200
ns
*
**

Figure 35. FM induced cytokine and chemokine production in mouse macrophage cell line after 24h co-culture.

Macrophage cell line RAW264.7 was primed with 10ng/ml LPS for 3h, then cultured in DMEM with 10% FBS culture medium overnight and on the second day, co-cultured with free mitochondria (FM, 100μg/ml) or mitochondrial lysate (MTDs, 100μg/ml). FM400 μg/ml is relative to the mitochondria released by a 2.5% liver injury and MTDs 100μg/ml is relative to the mitochondria released by a 10% liver injury. (A) IL-1β, (B) IL-6, (C) MMP9, (D) CXCL1, (E) VEGF-A, (F) IL-13, (G) CCL5 and (H) CCL4. n=3 per group. *, $P< 0.05$; **, $P<0.01$, ****, $p<0.001$.

Figure 35


A
IL-1β
B
IL-6
C
MMP9
D
CXCL1
E
VEGF-A
F
IL-13
G
CCL5
H
CCL4
pg/mL
Mock
FM100
MTD100
****
*
**

3.3.4 FM induce inflammatory responses in KC

Having demonstrated that both a mouse macrophage cell line and primary bone marrow-derived macrophages could be activated by FM. I next sought to determine whether extracellular mitochondria could activate KC. Primary mouse KC were isolated from C57BL/6 mice and purified them using Anti-F4/80 MicroBeads UltraPure (Miltenyi Biotec, CA) (Figure 36). The purified KC were used for co-culture experiments. The primary KC were co-cultured with media alone, FM or MTDs, and in addition, LPS was used as an activation control. Consistently, KC were activated in response to FM but not MTDs, and significantly induced IL-1β, MMP9, CXCL1, CCL2, IL-13 and CCL5 production (Figure 37). Surprisingly, FM and MTDs both suppressed IL-6 and CCL3 production in KC (Figure 37B and C). As controls, LPS-stimulated KC secreted pro-inflammatory cytokine and chemokines including IL-6, CCL3, CCL4 and CCL5 (Figure 37).

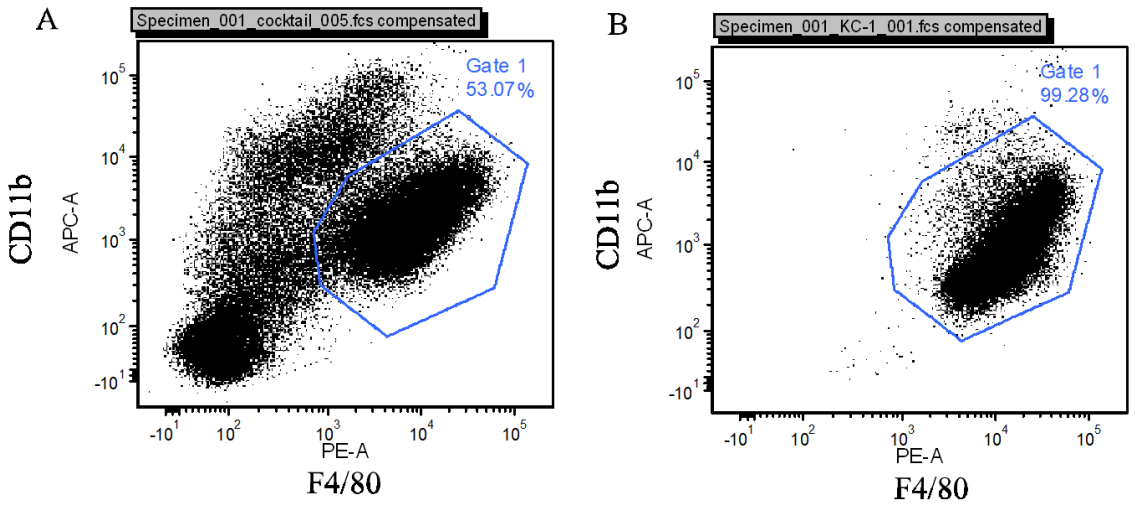


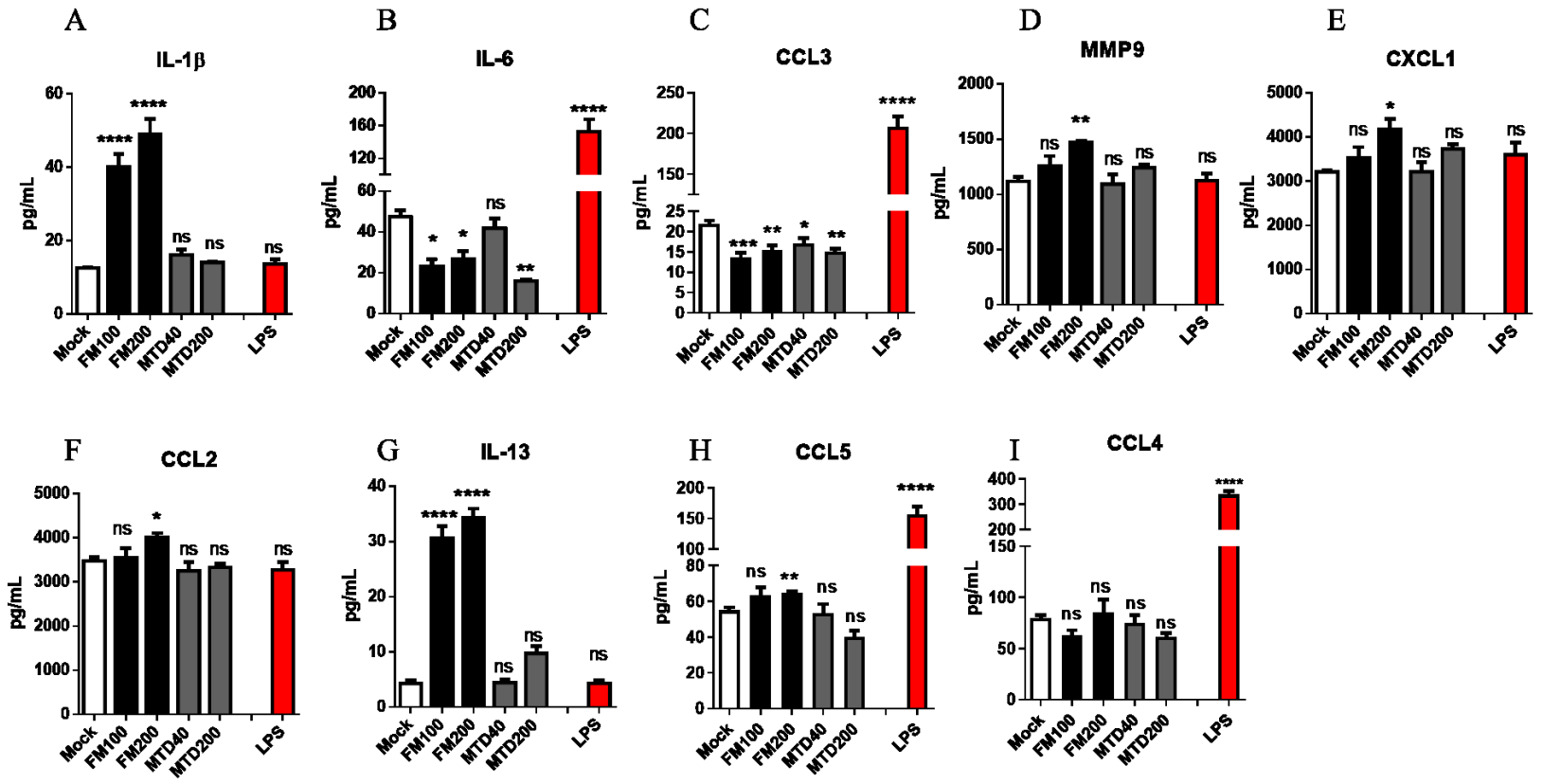
Figure 36. The purity characterization of primary Kupffer cells identified by F4/80^{+}CD11b^{+} non-parenchymal cells isolated from mouse liver.

Non-parenchymal cells (A) were separated from mouse live by Percoll® PLUS (Sigma, Canada), mouse KC (B) were identified by anti-mouse F4/80 and anti-mouse CD11b antibodies.

Figure 37. FM induce cytokine and chemokine production in mouse primary KC at 24h co-culture.

Mouse primary KC were co-cultured with free mitochondria (FM, 100 and 200μg/ml) and mitochondrial lysate (MTDs, 40 and 200μg/ml). FM200 μg/ml is relative to the mitochondria released by a 1.25% liver injury and MTDs 40μg/ml relative to the mitochondria released by a 4% liver injury. (A) IL-1β, (B) IL-6, (C) CCL3, (D) MMP9, (E) CXCL1, (F) CCL2, (G) IL-13, (H) CCL5, and CCL4. n=3 per group. *, $P< 0.05$; **, $P<0.01$, ****, $p<0.001$.

Figure 37


A
IL-1β
B
IL-6
C
CCL3
D
MMP9
E
CXCL1
F
CCL2
G
IL-13
H
CCL5
I
CCL4
pg/mL
Mock
FM100
FM200
MTD40
MTD200
LPS

3.3.5 FM activate macrophages *in vivo* independent of FPR1

Having demonstrated a role for macrophages in response to extracellular FM *in vitro*, whether macrophages could be activated by FM *in vivo* was investigated. Given that FPR1 played an important role in the MC responses to FM *in vitro* and *in vivo*, the effects of the FPR1 inhibitor (CsH) was also included in this macrophage study. As shown in Figure 27 D and E *in vivo*, the number of resident macrophages (named "resting macs") was significantly decreased in response to FM and the infiltrated monocytes/macrophages (named "inflamm macs") were significantly increased. Moreover, CsH prevented monocyte infiltration, but it did not affect the changes in the number of resident macrophages. Next, by using anti-mouse CD69 (signal transducing receptor) and anti-mouse CD11c (endocytic receptor) antibodies, we explored how resident and infiltrated macrophages responded to FM *in vivo*. FM could significantly induce CD69 expression in inflammatory macrophages and although the expression of CD69 on inflammatory macrophages was partially inhibited by CsH pre-treatment, the expression level was still significantly higher than vehicle control (CsH+PBS) (Figure 38A). The expression of CD11c was not altered by FM treatment of inflammatory macrophages (Figure 38B). The expression levels of CD69 and CD11c on resident macrophages were significantly upregulated by FM but FPR1 inhibitor CsH could not attenuate the expression levels of these two activation markers on resident macrophages (Figure 38 C and D). Representative staining plots with percentage changes are shown in Figure 38 E. Overall, these data indicate that FM activated resident macrophages and infiltrating macrophages *in vivo*, however, these activations were not attenuated by inhibition of FPR1 receptor function.

To confirm the apparent lack of inhibition of FPR1 on macrophages in response to FM, we further examined cytokine and chemokine production by macrophages *in vitro* by examining in macrophage supernatants with and without CsH pre-treatment in response to PBS or FM. Table 5 and Table 6 summarize macrophage cell line and bone marrow-derived macrophages cytokine and chemokine production in four groups. Consistent with *in vivo* data, CsH could not impair inflammatory mediator production by macrophages in response to FM, whereas the vehicle control groups were at similar level. Overall, these data strongly support our *in vivo* findings that the activation of macrophages induced by FM was not substantially dependent on the FPR1 receptor.

Figure 38. FM induce macrophage activation *in vivo* in a FPR1-independent manner. C57BL/6 mice were injected i.p. with PBS or FM (5mg/kg) (equal to the mitochondria released by a 2.5% liver injury), or pre-injected with FPR1 inhibitor, Cyclosporin H (2mg/kg) 30 min before FM injection. After 16 hours, the peritoneal contents were harvested by lavage, total peritoneal cavity cells counted and the percentage of CD69 positive and CD11c positive resting and inflammatory macrophages in each sample identified by flow cytometric analysis. (A) $CD69^+$ inflammatory macrophages, (B) $CD11c^+$ inflammatory macrophages, (C) $CD69^+$ resting macrophages, (D) $CD11c^+$ inflammatory macrophages, (E) Staining showing the phenotype of inflammatory and resting macrophages for PBS and FM injected C57BL/6 mouse with or without CsH pre-treatment groups. The graphs shown are the mean±SEM, n=5 per group. *, $P< 0.05$; **, $P<0.01$; ***, $P<0.0005$; ****, $P< 0.0001$

Figure 38

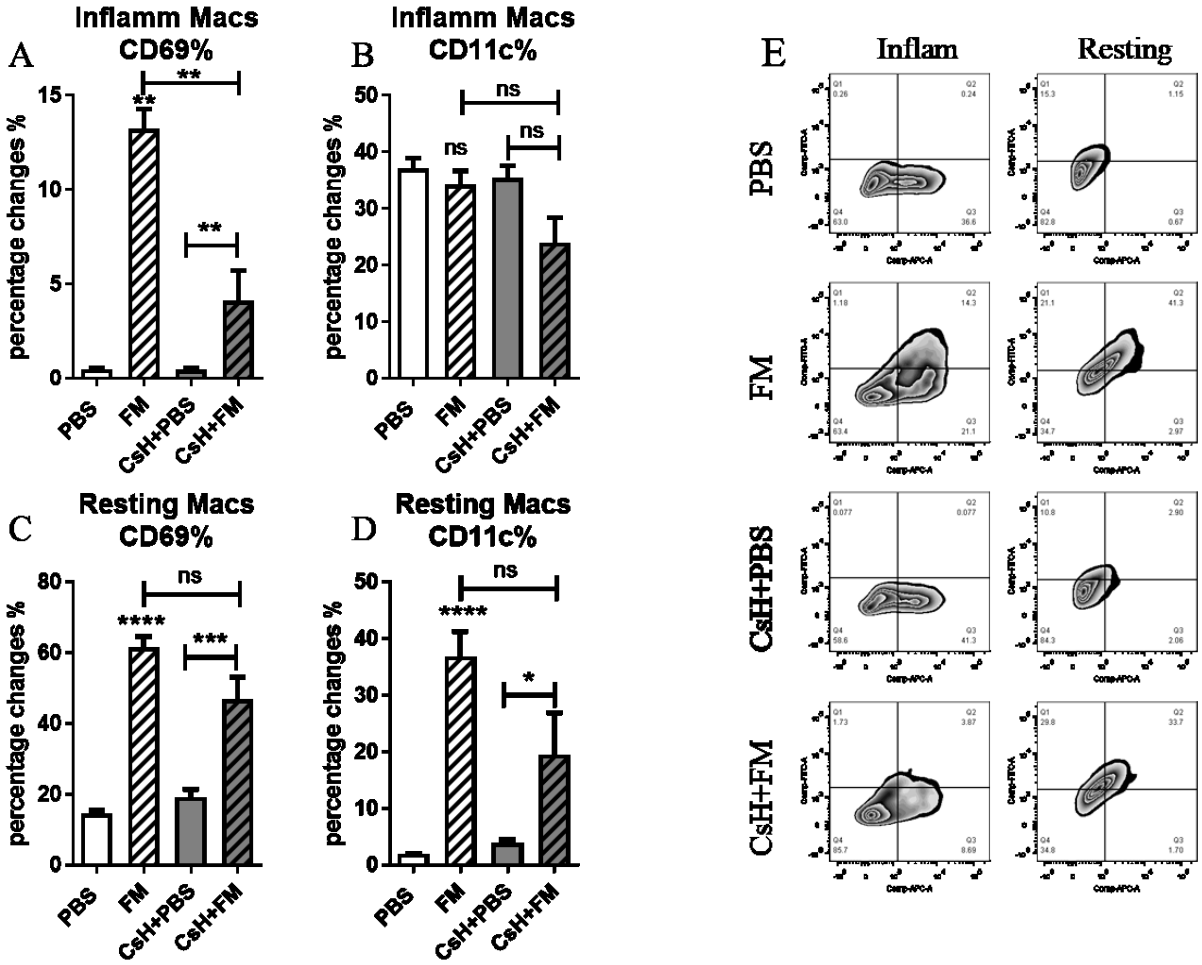

A
Inflamm Macs
CD69%
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Table 5. RAW264.7 macrophage cell line activation in response to FM is FPR1-independent.

	IL-1β	IL-6	MMP9	CXCL1	VEGF-A	IL-13	CCL5
PBS	24.86±0.4422	16.1±3.911	5795±40.23	1.00±0.0529	20.51±0.3626	4.390±0.1563	10221±164.9
FM	80.06±3.015	49.17±7.819	7183±56.77	31.49±1.926	42.38±0.8400	90.46±2.970	12397±184.2
CsH+PBS	27±0.8653	8.567±2.133	5739±23.75	4.927±1.987	20.94±0.2900	4.520±0.04163	9347±65.15
CsH+FM	81.16±5.221	63.73±10.26	8388±30.49	40.49±1.980	48.16±0.7503	90.46±2.573	13492±61.85

Cells were co-cultured with PBS or free mitochondria (FM) with or without CsH pre-treatment. Concentration of each mediators (pg/ml) was detected in the supernatant at 24h of co-culture.

Table 6. Mouse BMDM activation in response to FM is FPR1-independent.

	IL-1β	IL-6	CCL3	MMP9	CXCL1	CCL2	IL-13	CCL5
PBS	354.9±36.67	364.8±20.21	15.39±2.317	252.2±5.998	405.9±16.96	4439±535.1	168.3±6.702	2737±91.91
FM	472.2±20.26	508.2±48.71	24.52±0.8182	445.0±8.578	555.9±33.38	7187±757.7	203.0±9.568	3588±58.31
CsH+PBS	310.5±52.26	349.5±82.81	20.59±4.221	254.4±5.265	301.2±78.53	3823±906.8	164.4±7.399	2752±496.3
CsH+FM	519.3±21.83	1054±39.78	47.59±1.790	472.7±14.73	976.2±13.44	12614±410.3	207.0±11.52	6976±174.9

Cells were co-cultured with PBS or free mitochondria (FM) with or without CsH pre-treatment. Concentration of each mediators (pg/ml) was detected in the supernatant at 24h of co-culture.

CHAPTER 4 DISCUSSION

4.1 Summary of major findings

IRI during liver transplantation enhances the immune response to allografts, promotes inflammation and thus impacts overall graft outcome. This sterile inflammatory insult is known to activate innate immunity and propagate organ damage through the response to endogenous alarmins. The current study demonstrates convincingly, for the first time, that extracellular mitochondria, similar to other endogenous alarmins such as HMGB-1 and nuclear fragmented DNA, can contribute to hepatic IRI and may serve as biomarkers of liver IRI. Co-culture of mitochondrial components with hepatocytes and macrophages showed a decrease in cell viability in a concentration dependent manner which indicates that extracellular mitochondrial components are toxic mediators participating in the pathogenesis of liver IRI. Furthermore, our data provides evidence that extracellular mitochondria play an active role in enhancing innate immune cell activation, specifically of MC and macrophages.

Given the role of extracellular mitochondria in murine cells, primary human cells or *in vivo* rodent models, I questioned whether my findings could be verified in human liver transplantation. To this end, I performed gene copy number and expression analyses on datasets from clinical studies in the human liver transplantation database, which contrasted global gene expression profiles obtained from peripheral blood of tolerant and non-tolerant recipients [321]. Of note, I identified the significant up-regulation of CXCL8, CCL3 and IL-1β (Figure 39) in non-tolerant liver transplant patients compared with the tolerant liver transplant patients, which suggested that extracellular mitochondria might contribute graft rejection in liver transplant patients. While the *in vivo* models used in this

study might not be an optimal source to dissect the molecular mechanisms responsible for liver injuries, the use of a local inflammation model might be beneficial for exploring the regulation of extracellular mitochondria mediated immune responses during the initiation or progression of human disease. These diseases include acute graft failure or chronic graft versus host disease in transplantation, rheumatoid arthritis, or in patients with blood transfusion, which have been reported to demonstrate FM release.

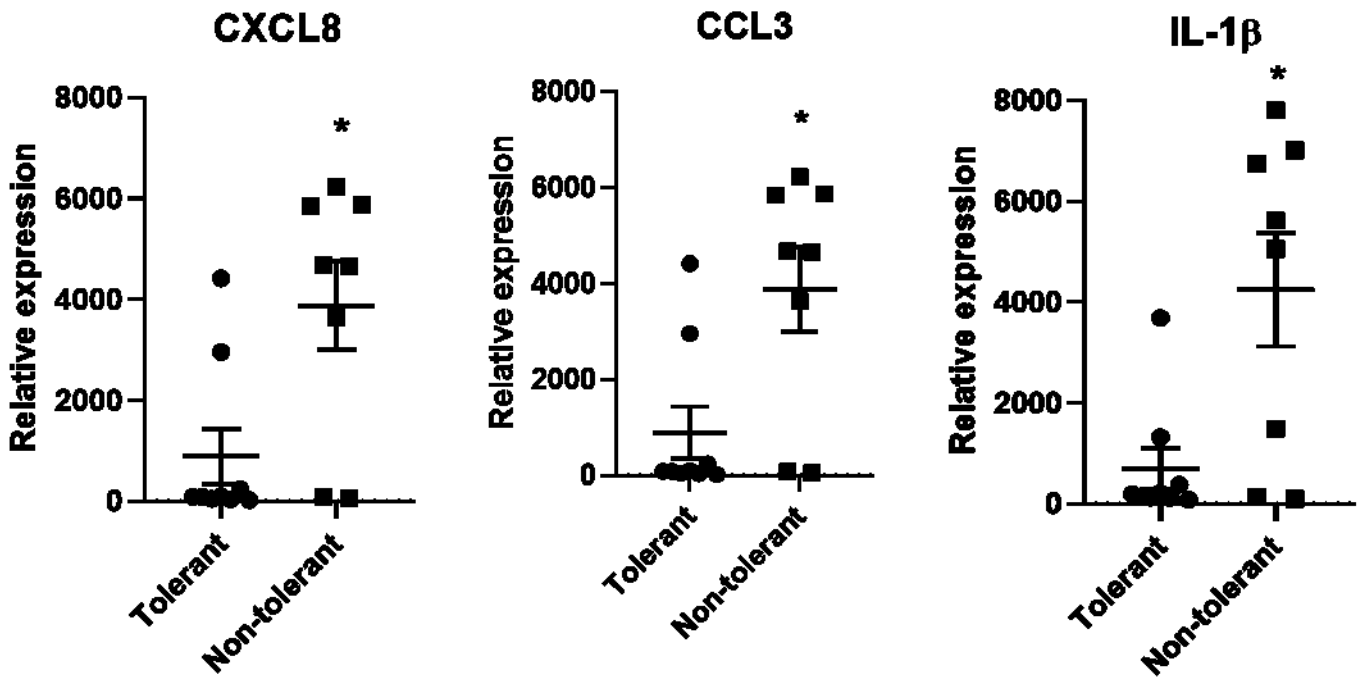


Figure 39. Up-regulation of pro-inflammatory genes between tolerant and non-tolerant recipients. Tolerant recipients of adult deceased donor liver transplants (>1 year of successful immunosuppression discontinuation) (n = 9), immunosuppression dependent recipients (Non-tolerant) (n = 8). Clinical characteristics of patients included in the study are summarized in [321].

4.2 Implications and relevance of major findings

4.2.1 Extracellular mitochondria as predictor markers and therapeutic target for liver IRI

There is a growing body of literature suggesting that mitochondrial alarmins are implicated in various human diseases, and the measurement of mtDNA in human biological fluids provide an assessment of mitochondrial alarmins suggesting their potential use as biomarkers for many diseases. In the current study, interestingly, different patterns of mtDNA release were observed when cells were subjected to transplantation-related and warm ischemia. The elevation of mtDNA was markedly higher in the IR compared with cold ischemia incubation alone in two hepatocyte cell lines (Figure 6). In contrast, after warm ischemia, the levels of mtDNA increased immediately both *in vitro* and *in vivo*, and these high levels were maintained in the reperfusion phases (Figure 7 and Figure 12). These results indicate that different mechanisms of injury are likely involved in cold and warm hepatic IRI. This is consistent with previous studies demonstrating that in cold ischemia, during hypothermic preservation, toxic mediators are not released [322], whereas during warm ischemia, these toxic mediators are up-regulated [323]. The increase of mtDNA after reperfusion suggests not only that warm ischemia induced mitochondrial alarmin elevation, but that reperfusion further triggered additional mitochondrial component release.

Machine perfusion is rapidly developing in transplantation. Data indicate that machine liver preservation is superior to conventional cold storage and gave a window to assess the tissue damage and organ function before implant into the recipient [324]. Previous researches identified multiple alarmins derived from nuclear or cytosol to be used as predictive markers of graft viability in the perfusate, which also included mitochondria products such as mitochondrial ROS and ATP production into the perfusate [325]. Using

mtDNA or quantification FM as biomarkers to predict the liver transplant out come during machine perfusion might be beneficial for liver transplantation outcome. Additionally, providing supplements to the perfusate to prevent mitochondrial damage, clear or degrade mitochondrial alarmins or inhibit the inflammation induced by potential mitochondrial alarmins might help to control tissue damage and optimize organ function before transplant.

We have further demonstrated that mitochondria lysates (MTDs) trigger apoptosis and necrosis in a dose dependent manner *in vitro* (Figure 8 and Figure 9). These results suggest that MTDs may serve as a toxic mediator participating in the pathogenesis of liver IRI. Zhang *et al* . [82] reported that severe trauma releases mitochondrial alarmins concluding mtDNA and MTDs, into circulation and that mtDNA was further elevated 24h after injury. These so-called MTDs were found to activate neutrophil migration, and circulating MTDs were suggested to elicit neutrophil-mediated organ injury [82]. Previous studies of liver IR also found that the late phase of IRI involved migration of neutrophils in response to release of toxic mediators, such as ROS and other alarmins [323]. Based on our results, we hypothesize that mitochondrial components might be one of the cellular alarmins that play a role in neutrophil activation and in later neutrophil-mediated hepatic IRI. We found that when hepatocytes were co-cultured with different concentrations of the MTDs, the percentage of apoptotic cell death decreased, whereas the percentage of necrotic cell death increased in a dose-dependent manner. We also found that the level of TNF was up-regulated in hepatocytes co-cultured with MTDs, which was 16.12±2.974 fold higher than the control group ($P<0.001$) (Figure 11 and Table 2). It has been demonstrated that stimulation with TNF can induce either apoptosis or necrosis, and that the late stage of apoptosis is associated with secondary necrotic cell death [42]. These findings suggest that

MTDs can induce to both apoptosis and necrosis in hepatocytes, and that late apoptosis might be associated with necrotic cell death in association with TNF elevation.

Although previous studies have investigated the production of inflammatory cytokines in liver IR models, most of them detected these cytokines at 6h or 24h post-reperfusion, mainly related to the infiltration of white blood cell in the late phases of liver IRI [296, 326-328]. In our study, we found that there was a marked increase of IL-6 and IL-10 expression just one hour post-reperfusion, during the early phase of liver IRI without neutrophil or monocyte infiltration [323]. The release of inflammatory cytokines in our experiments was likely related to the activation of resident liver immune cells, such as MC, macrophages or DC. Since we demonstrated that MTDs can trigger apoptosis and necrosis *in vitro* (Figure 8), which may also play a role in the activation of KC or DC to induce an acute inflammatory response. Moving forward, we wanted to determine whether MTDs can contribute to activating immune responses that lead to inflammation.

Previous studies have shown MTDs can cause neutrophil-mediated organ injury *in vivo*, which is associated with significant TNF and IL-6 secretion [23]. Numerous studies have shown that TLR4 and TLR9 receptors participate in liver IRI models *in vivo*, as well as KC models *in vitro* [43-47]. A recent study also demonstrated that MTDs accumulate in the liver, kidneys, lungs, and lymph nodes following tail vein injection, and can induce IL-1β and IL-6 secretion as well as up-regulate numerous pro-inflammatory genes in the liver [135]. These studies suggest that one of the mechanisms of hepatocyte cell death in our hepatic IR model may be through MTD induction of immune cells. This may include neutrophils, liver resident KC, DC, infiltrating monocytes and other immune cells causing inflammation by secreting pro-inflammatory cytokines. We show clearly in our study that

hepatocytes co-cultured with MTDs undergo cell death similar to IR (Figure 8). Expression of mRNA for *TLR2*, *TLR4*, *TLR9*, *MyD88* and *NFκB* were similar for hepatocytes co-cultured with 400μg/ml MTDs, warm IR and cold IR treatments (Figure 10). The expression levels of all these genes were significantly increased in hepatocytes co-cultured with MTDs, which was similarly observed during the warm and cold IR treatments when compared to the control group. In this model, we report for the first time that MTDs induce MyD88 and NFκB expression and up-regulate the expression of TLR2, TLR4 and TLR9 and TNF production in the hepatocytes (Figure 10 and 11).

In conclusion, the first part of my project suggested that hepatic IR results in a significant increase of extracellular mitochondrial alarmins, represented by mtDNA and intact mitochondria, both *in vitro* and *in vivo* suggesting that mtDNA may serve as a novel marker in hepatic IRI. Co-culture of MTDs with hepatocytes showed a decrease in cell viability in a concentration dependent manner, which indicates that MTDs are a toxic mediator participating in the pathogenesis of liver IRI. This study illustrates a novel marker for hepatic IRI and potentially provides additional targets to ameliorate the sterile inflammatory response associated with ischemia and reperfusion injury.

4.2.2 The role of MC in response to extracellular mitochondria

Mitochondrial components secreted from cells can act as endogenous alarmins, a term first coined by Zhang *et al* . [82]. Since they bear bacteria-derived molecular motifs, extracellular mitochondria can provide both “sterile” and “infection-associated” danger signals. The release of mitochondria from injured cells results in a wide variety of inflammatory events. Intact FM can be passively released from necrotic cells or TNF-induced necroptotic cells or during extracellular traps formation of neutrophils, MC or

eosinophils [329]. Indeed, mitochondrial components are involved in multiple disease processes including sepsis [330, 331], stroke [332], pulmonary embolism [333], acute liver failure [69], rheumatoid arthritis [334], myocardial infarction [335], graft-versus-host disease [134, 336], asthma [337], pneumonia [338], and cancer [339-341]. Among these studies, intact FM proved to be major sources of mitochondrial alarmins in non-alcoholic steatohepatitis patient [90], diseased donor blood [134], transfusion products [135, 342] and were detected in rheumatoid arthritis patients [135, 329]. Quantification of mtDNA in patient fluid by qPCR or detection of intact mitochondria by flow cytometry was shown to be clinically useful to predict prognosis or response to therapy [72, 330, 343, 344].

Recent studies demonstrated that intact mitochondria as an extracellular organelle could be a significant source of mitochondrial alarmin signals, which may contribute neutrophil NETosis or activate EC [134, 135]. Our study is to first investigate how FM activate resident innate immune cells and induce local inflammation and cell recruitment. We have shown that FM can evoke acute inflammation by recruitment of innate immune cells, including neutrophils, inflammatory macrophages, DC, MC, eosinophils and NK cells (Figure 20 1A-N) *in vivo*. This finding is concordant with previous studies demonstrating that mitochondrial components, such as mtDNA and formyl peptides, promoted the transmigration of neutrophils, leading to distant organ inflammatory responses [82, 345]. In addition, some mitochondrial components mediate macrophage and DC [346-348] trafficking and activation. In these cases, mitochondria were the main cellular producers of inflammasome activators [91, 349-351], and mitochondrial components were elevated within the immune cells rather than released into the extracellular milieu.

To examine how FM impact the initiation of innate immune responses, we investigated MC, which are important immune sentinel cells with a wide variety of pattern recognition receptors. Due to their strategic location at mucosal surfaces and in the skin, they are key cells in surveillance against pathogens and tissue damage. A key role for MC in innate responses is initiating recruitment of innate immune effector cells. Our study in MC deficient mice demonstrated that MC were essential for the recruitment of neutrophils (Figure 21F), DCs (Figure 21I-K), and eosinophils (Figure 21L) in a model of FM triggered peritonitis. This study is consistent with previous studies, which indicate that MC are crucial for neutrophil, eosinophil and macrophage recruitment in models of bacterial infection, and promote anti-microbial and anthelmintic functions of these cells [258, 352, 353].

MC not only coordinate the influx of cells that subsequently eliminate dangerous stimuli but are themselves effector cells in innate immune responses. They are capable of phagocytosis and release a unique panel of soluble mediators with pro-inflammatory, anti-inflammatory and/or immunosuppressive properties upon activation [354, 355]. We found that FM could induce both primary human and murine MC degranulation at a concentration range from 20μg/ml to 200μg/ml, while a higher concentration of FM (400μg/ml) failed to MC degranulation (Figure 25M and N). This 'bell-shaped' response curve pattern was also found for the CCL2, CCL3, CCL4, MMP9, CXCL1, CXCL8, and IL-6 responses when human CBMC were activated by human FM at 6h or 24h time points (Figure 25A-G). Interestingly, a similar trend was found in neutrophils responding to mitochondrial lysates/MTD's, which showed desensitization and suppression of CXCL8 production by high concentrations [82]. These responses might due to the variety of mitochondrial

components in FM that could induce both pro-inflammatory and anti-inflammatory mediators. Significantly higher levels of anti-inflammatory mediators, IL-1RA and IL-13 production was observed in CBMC responding to high concentrations of FM (Figure 25I and K), which could potentially limit pro-inflammatory mediator production in an autocrine manner. The mitochondrial components responsible for the MC degranulation have not yet been identified. We observed that mtDNA, MTDs or formyl peptides which did not induce MC degranulation effectively alone (Figure 25). Multiple mitochondrial components or signals associated with intact FM may be required to induce this degranulation process. Notably, neutrophils are not activated in response to CpG DNA, mtDNA, or the synthetic peptide N-formyl-Met-Leu-Phe (fMLF) alone [82]. However, MTDs are potent inducers of neutrophil degranulation (observed by production of MMP8) and activation (observed by production of CXCL8). In contrast, neither MTDs or mtDNA could induce CXCL8 production in CBMC (Table 4 and Figure 24). However, MTDs induced significant IL-1RA, IL-13, and VEGF-A production in a dose-dependent manner (Figure 24) and mtDNA induced slight IL-1β elevation at 6h (Mock: 5.767± 1.356 pg/ml versus mtDNA: 15.02± 2.896 pg/ml) in CBMC. Responses to MTDs by MC induced less inflammatory cytokine production and wound healing-related growth factor production, indicating a possible role in reducing long term inflammation and promoting tissue remodelling when MC respond to high local concentrations of mitochondrial alarmins.

MC activation was also detected in our *in vivo* model to measure responses to FM. We found that there were significant increases in the percentage and number of $CD69^+$ MC in the peritoneal cavity upon FM administration compared with vehicle control (Figure 22A-C), which were associated with significantly increased local levels of MMP9, CXCL1,

CCL5 and IL-4 (Figure 22D-G). Moreover, this increase of the aforementioned cytokines was abolished in MC deficient mice in response to FM (Figure 22I-L). We could not conclude that all these mediators were directly produced by MC upon activation *in vivo*, however, by co-culture of CBMC with FM *in vitro*, we observed significant increases of MMP9 and CXCL1 (Figure 25D and E), which demonstrates that MC have the ability to secrete those mediators in response to FM challenge. Previous studies also reported MMP9, CXCL1, CCL5 and IL-4 secretion upon MC activation in mouse models of infection and inflammation [356-359]. They play an important role in the recruitment of neutrophils [357, 360] and eosinophils [260, 359], while enhancing activation of macrophages [260] and as a positive feedback mediator to activate MC themselves [356]. Strikingly, our experiments found significant increases of IL-4 production in our *in vivo* model. Aside from MC, the peritoneal cavity contains mainly B1 lymphocytes and resident macrophages under steady state conditions, neither of which secrete IL-4. Of note, MC are essential for IL-4 production in mouse peritoneal cavity [361], therefore the elevation of IL-4 in MC sufficient mice versus alleviation of IL-4 production in MC deficient mice in response of FM could represent the activation of MC in our *in vivo* model (Figure 25G and L). Overall, the present study showed that MC are activated by FM *in vivo* and play a crucial role in the secretion of MMP9, CXCL1, CCL5 and IL-4 in response to FM.

MC sense inflammatory stimuli and their surrounding environment via a plethora of cell surface receptors [260]. Interestingly, our results showed that the degranulation pattern of CBMC in response to FM was both rapid and independent of extracellular calcium (Figure 25O and P). These data are consistent with a GPCR dependent process [268]. The requirement for several well-known receptors, including GPCR's for

mitochondrial alarmins, such as P2X7, TLRs, and formyl peptide receptors [69, 82, 318, 319, 362] were investigated. Of the receptor blockade strategies we used, only the FPR1 inhibitors (CsH and Boc-1) effectively attenuated the MC activation response to FM (Figure 26D, E, G, H and I). Blocking P_2X_7 or TLRs did not affect MC activation in response to FM (data not shown). Previous studies have defined FPR1 intracellular signaling events, including roles for phosphoinositide-3 kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPKs) [363]. We found that ERK/p38 and IκB kinase (IKK) inhibitors significantly attenuated MC activation, which was triggered by FM (Figure 40A and B) while JNK, Akt or mammalian target of rapamycin (mTOR) inhibitors (Figure 40C-F) had little impact. These results were consistent with a previous study, which observed that inhibition of p38 MAPK could protect extracellular mitochondria triggered adverse reactions [364].

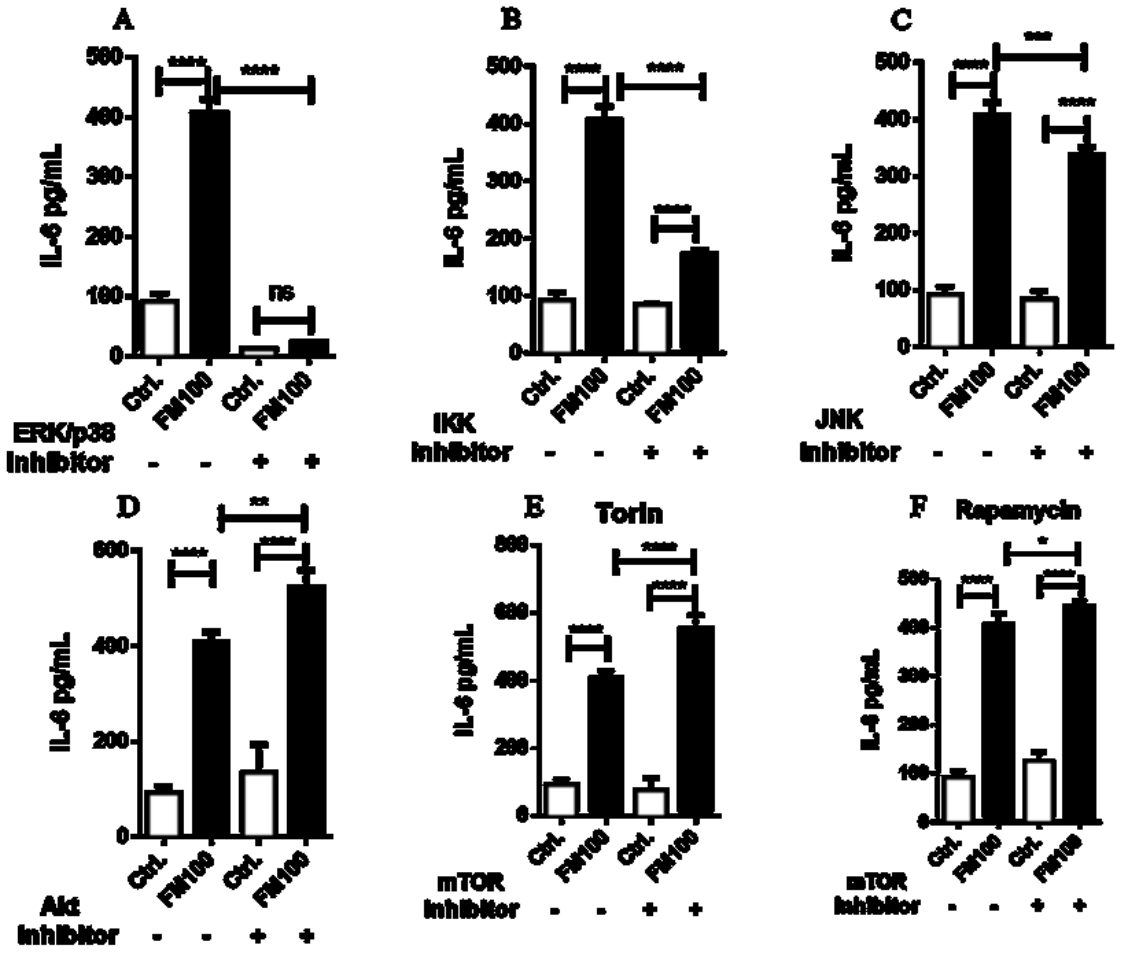


Figure 40. Potential signaling pathways in FM induced inflammatory responses. BMMC were used to co-culture with or without different signaling pathway inhibitors. IL-6 production was used as functional readout. Inhibitors for ERK/p38, NFκB and JNK significantly decreased the IL-6 production in BMMC supernatants, but not Akt or mTOR inhibitors. *, P< 0.05; **, P<0.01; ***, P<0.0005; ****, P< 0.0001

The role of FPR1 in response to FM was also examined *in vivo*. MC numbers and the proportion of these cells that were CD69+ were not elevated when mice were pretreated with the FPR1 inhibitor (CsH) 30min before FM injection (Figure 27J and K). The responses of other innate immune cells, such as neutrophils, inflammatory macrophages, DC, and eosinophils (Figure 27) were also decreased. *In vitro* blockage of FPR1 reduced both human and mouse MC CCL3 and CXCL8 production in CBMC (Figure 26D and E) and IL-6 production in BMMC (Figure 26G), suggesting that despite impacts on other cell types, the activation of FPR1 in MC is an important response pathway during FM release. Taken together, we suggested that both MC and FPR1 are important to amplify the inflammatory process triggered by FM. Additional work using a combination of human samples and animal models will be required to define the role of FM and FPR1 in driving inflammation via other immune cells.

In summary, we propose that FM released during tissue damage or infection can promote inflammatory responses by activation of tissue resident MC, initiating a process that will recruit and activate other innate immune cells (Figure 41). In addition, FM may activate other cell types by both FPR1 dependent and independent mechanisms. Taking into account our mouse models and *in vitro* MC activation data, we propose that controlling MC activation, or the use of FPR1 inhibitors, might be promising alternatives to reduce inflammatory responses in patients with predominant extracellular mitochondria release such as liver transplantation patients or patients repeatedly transfused with extracellular mitochondria. Our results demonstrate an important role for MC in evoking and amplifying the inflammatory process triggered by FM. Although FM can promote pro-inflammatory responses in various experimental models, their roles in human diseases may be

multifunctional and complex. Further studies of the mechanisms by which FM impact the initiation and progression of human diseases will be important to evaluate their role and the importance of MC and FPR-1 in relevant disease settings.

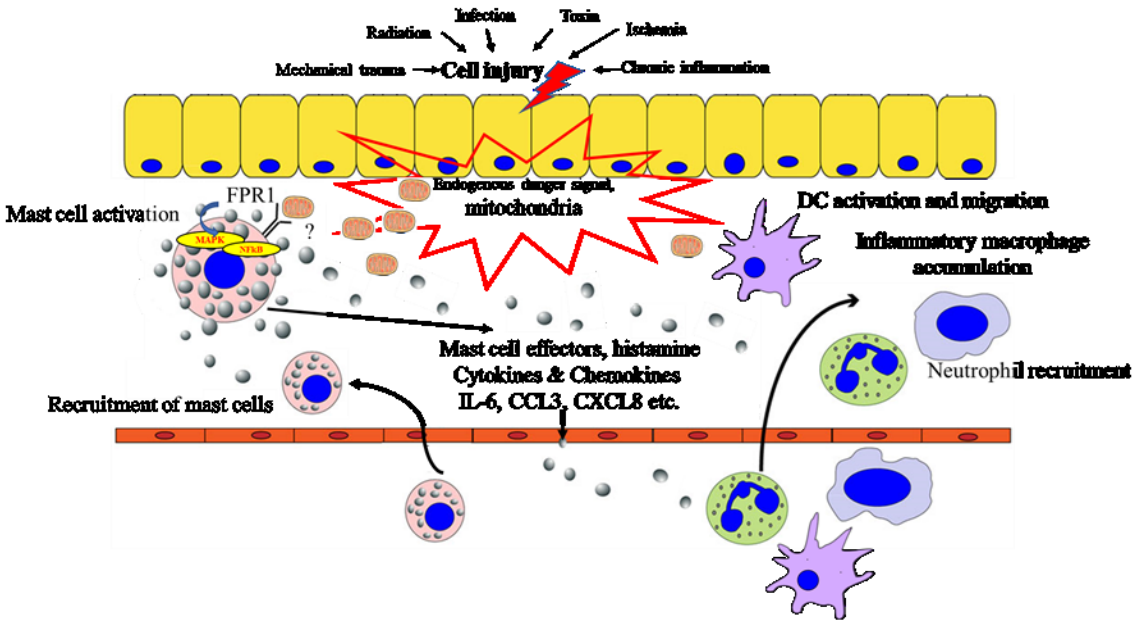


Figure 41. The effects of MC activation on the inflammatory environment induced by cell/tissue injury.

Within the tissues, MC can be activated by endogenous danger signals, such as FM resulting in the secretion of effector molecules. MC recognize FM resulting in MAPK and NFκB activation and, eventually, the subsequent transcription of several pro-inflammatory genes. Release of cytokines, chemokines, and other effector molecules together initiates an inflammatory response, for instance, resulting in neutrophil recruitment, accumulation of inflammatory macrophages, activation and migration of DC. This pro-inflammatory response initiated by MC responding to FM can be attenuated by FPR1 inhibitors. MC accumulation in the damaged tissues could be due to either the recruitment and differentiation of MC progenitors to the infected tissue and/or proliferation of the tissue-resident MC population.

4.2.3 The role of macrophages in response to extracellular mitochondria

Due to their great abundance in liver, hepatocytes are the main source of mitochondria during liver injury. Thus, the presence of mitochondria in the extracellular milieu in injured liver, such as IRI liver (Figure 17), is highly likely to activate hepatic resident immune cells. Studies were, therefor, undertaken to determine the impact of hepatic immune cell activation on mitochondria release. The liver harbours the greatest density of macrophages among all solid organs in the body [365]. Macrophages play an important role in phagocytosis of dead cells or cellular debris and clearance of endogenous alarmins [122, 366]. KC are a self-sustaining, liver-resident population of macrophages and can be distinguished from the monocyte-derived macrophages that rapidly accumulate in the injured liver. KC and monocyte-derived macrophages rapidly adapt their phenotypes in response to local signals, which determine their ability to aggravate or limit liver injury [367]. We first investigated how the different subsets of macrophages response to IR, particularly focused on how their mitochondrial homeostasis was disturbed during IR. Previous studies suggested that macrophages produced reactive free radicals, such as ROS, which led to swelling of sinusoidal EC and hepatocytes injury [368]. Our former study found that during IR, mitochondria are a major source of ROS during the ischemia and reperfusion processes in macrophages (Figure 5A). Furthermore, we found that M1 and M0 were more susceptible to IRI, which led to more serious mitochondrial damage and potential cell death (Figure 30 and Figure 31), however, we are not sure if the IR process acting on macrophages themselves would contribute their phenotypic changes. Nevertheless, previous studies had shown that an acute inflammatory environment in injured liver would contribute to driving liver macrophages towards an M1 phenotype,

whereas, in the resolution phase, macrophages developed into an M2 phenotype mediated by IL-4 and IL-10 secretion and contributed to subsequent healing and resolution [366]. Furthermore, we observed that extracellular mitochondrial alarmins did not directly induce macrophage phenotypic changes; however, extracellular mitochondria could induce macrophage activation by co-culture. They could enhance pro-inflammatory mediator production in M1 macrophages; whereas, for KC, FM could induce both pro-inflammatory mediators such as IL-1β, MMP9, CXCL1, CCL2, and CCL5, and anti-inflammatory cytokines such as IL-13. Interestingly, following co-culture with FM, KC also showed a decline of IL-6 and CCL3 production (Figure 37). These findings were consistent with previous studies which had rationalized that KC have dual regulatory actions in liver diseases [367, 369, 370].

To investigate how macrophages respond to mitochondria *in vivo*, we injected FM into the mouse peritoneal cavity to examine both the resident and infiltrating macrophages at the local site of injected FM. Interestingly, we found that FM could increase the activation marker CD69 expression on both resident and trafficking macrophages, whereas only CD11c (endocytic receptor) expression was elevated on the resident macrophages, which indicated that the resident macrophages might uptake the naked organelle FM. These findings were consistent with a previous study, which showed that resident lung macrophages could readily acquire mitochondria from mesenchymal stromal cells *in vivo* and display more pronounced phagocytic activity in a model of acute respiratory distress syndrome and sepsis, thereby improving bacterial clearance [371]. Thus, FM might also modulate macrophage phagocytic activity on resident macrophages in our model, which contributed further to alarmins clearance. Surprisingly, both the activation of macrophages

in vitro and *in vivo* was independent of FPR1 receptor, which indicated there might be other mechanisms to activate macrophages by FM, distinct from those used by MC. Based on the elevated expression of the (endocytic receptor) CD11c, macrophage phagocytosis might play a role in activation induced by FM. This was also supported by a recent study that DC could uptake extracellular FM and inhibitors of phagocytosis including poly-I (class A scavenger receptor inhibitor) and cytochalasin E (inhibitor of actin polymerization) could significantly prohibit mitochondria uptake [285].

4.3 Limitation of experimental systems

4.3.1 mtDNA versus FM

Currently, the most well described mitochondrial alarmin is mtDNA [372-374]. mtDNA can be released into the circulation due to cell damage or stress, and can also be released passively along with mitochondria, actively extruded in exosomes or extruded from granulocytes in the production of extracellular traps [375-377]. Our original studies used mtDNA as a marker for freely circulating mitochondrial alarmins during liver IRI. Although we observed FM in the IR liver biopsy, whether the quantity of mtDNA correlated with the amounts of FM, or if the levels of mtDNA were correlated with the liver damage in a clinical scenario is unknown. It is known that circulating mtDNA is employed as a feasible and powerful quantitative approach to measure the release of many other mitochondrial alarmins in the extracellular milieu [72]. However, the sources of circulating mtDNA and relationship to associated mitochondrial alarmin signals might need to be further confirmed if this is being used for therapeutic guidance.

As we have shown, co-culture of mtDNA with immune cells, including MC or macrophages did not promote such a massive pro-inflammatory response as we observed

when using FM. A previous study has shown that neither MTDs or mtDNA alone could induce neutrophils to secrete CXCL8 *in vitro*, in a sharp contrast, combining MTDs and mtDNA together could induce a robust CXCL8 production [82]. Similarly, in our study, we found that FM could potently activate MC and macrophages, which suggests that multiple pathways might synergize in the response to mitochondrial alarmins.

4.3.2 *In vivo* models

Few aspects of vascular pathobiology have garnered more attention over the past three decades than IRI [378]. Since it was first reported, a voluminous literature has accumulated implicating released endogenous molecules as important mediators and modulators of IRI in several different organ systems including the gut, liver, heart, kidney, brain and lung to mention just a few [379]. One organ system that has produced very detailed information regarding the mechanisms by which endogenous molecules modulate tissue damage following IR is the liver. It is well appreciated by the clinical community that liver IRI is a major complication associated with liver transplantation and re-sectional surgery as well as hemorrhagic or endotoxin shock and thermal injury [380-383]. In the area of liver transplantation, the shortage of organs has led centers to expand their criteria for the acceptance of marginal grafts that exhibit poor tolerance to IR [384]. Therefore, minimizing the adverse effects of IRI could improve outcomes in liver surgery, increasing the number of patients who successfully recover from major liver surgery. In this study, we used both global and partial hepatic IR model to investigate the mtDNA released during different stages of IR. The model of global liver ischemia with portal decompression ideally simulates the clinical situation of warm ischemia for liver resection and liver transplantation, which is associated with massive mesenteric congestion and represents

lethal model with severe bowel injury [385]. However, this model is difficult to approach in mice since it would lead to animal death during the ischemia phase. As a result, the partial hepatic IR model was used in mice to explore a longer term of reperfusion. Animal models of cold and warm hepatic IR are valuable tools for understanding the physiopathology of hepatic IRI and discovering novel therapeutic targets and drugs. However, a transplant model would be more optimal to further explore the role of IR in liver transplantation.

To explore how mitochondrial alarmins activate the immune system *in vivo*, we used a model of FM-triggered peritonitis. This model has advantages and disadvantages: it might not model effectively aspects of liver transplantation or liver IR; however, it gave us a broader application view of how FM modulate inflammation and leukocyte trafficking in a local inflamed site. We tested on the delivery of mitochondrial alarmins intravenously in rats, this treatment induced systemic inflammation, and most importantly, induced lung injury. This was consistent with clinical observations of pulmonary complications that occurred after liver transplantation [386-388]. However, we did not further pursue this intravenous injection model as we were more interested in examining the primary inflamed site of mitochondrial local impact. In liver transplantation following IR, the liver tissue would be the origin of the released alarmins and other inflammatory mediators. Systemic inflammation might be more likely a secondary inflammatory event during IRI. Since we have proven that large amounts of mitochondrial alarmins are released during liver IRI, further studies using a liver transplant model will be certainly helpful to understand the role of extracellular mitochondria in liver transplant and to evaluate strategies to block ensuing damaging inflammation.

4.3.3 Online database and human sample analyses

Although BMMC, BMDM and KC are primary cultured cells that are very similar to naturally developed MC or macrophages residing in the tissues respectively, they are still heterogenous populations. The cell line RAW264.7 was used to confirm our observation, but it is further away from resembling "real" physiological macrophages due to their transformative potential. We do appreciate primary CBMC in our MC study, which gave us a closer representation of how mitochondrial alarmins activate human MC. When pooling analyses of online datasets from human liver transplant databases, the human sample database for liver transplant is really limited and with very few patient samples available. To further validate my conclusions, new strategies to obtain and exam relevant human samples would be needed.

4.3.4 Mechanism exploration

In the present study, multiple inhibitors, antagonists, agonists, and antibody-neutralization methods were used to pinpoint potential receptors or pathways involved in responses to mitochondria or their products. The main reason for choosing pharmacological approaches to define potential signaling pathways is that extracellular mitochondria contain various molecules which might trigger multiple signaling pathways simultaneously and induce immune activations. Therefore, these pharmacological tools are one of the fastest and most convenient ways to determine which pathway(s) might be driver or critical for a response to mitochondrial alarmins. Further studies using knockout or knockdown models will be required to confirm our observations. It will be important in the next stage of studies in this area to investigate the signaling pathways leading to different types of immune cell activation by mitochondrial alarmins. The use of MC, macrophages

and KC from receptor and signaling pathway deficient mice and/or CRISPR based manipulation of human cells could be helpful in this regard.

4.4 Proposed future directions

4.4.1 FM uptake

While my study unmasked a role for activation of innate immune cells, e.g. MC and macrophages, in response to FM released by liver injury and its clinical implication, there are many questions remaining to be answered. The first question would be whether immune cells need direct contact with FM and uptake the naked organelle to induce inflammatory responses. If so, we would need to determine if mitochondrial uptake was dependent on scavenger receptors or actin polymerization. If not, we would investigate if mitochondria need to be degraded to expose internal molecules or if just certain surface molecules would be major alarmins to evoke immune system. It would be interesting to determine the dominant component(s) in mitochondria to activate inflammatory responses or to stimulate anti-inflammatory mediator responses.

Furthermore, most alarmins are themselves incapable of functioning as chemo-attractants. However, mitochondrial alarmins, such as mitochondrial formylated peptides have been shown to be important in recruiting neutrophils in models of sterile injury. In this model, massive necrotic cell death led to the release of large quantities of extracellular mitochondrial alarmins into the systemic circulation. It would be worthwhile to determine the chemotactic potential of products from FM and the potential for chemotactic responses of the organelles themselves.

4.4.2 MC reconstitution and additional MC-deficient models

Wsh mice are valuable tools for investigating MC *in vivo*. The c-kit/SCF axis, which is exploited by the Wsh model, is critical for the development of mature MC [389]. Introducing mutations in the gene *c-kit*, which is the receptor for SCF, results in mice devoid of mature MC. The $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ (Wsh) model of MC deficiency is commonly used [390-393] as this strain is fertile, not anemic, and has normal γδ T cell numbers compared to its predecessors [394]. However, *c-kit* is also expressed at low levels by a number of other cell types, such as basophils and interstitial cells of Cajal [394]. Therefore, *c-kit* mutations in these mice result in pleiotropic effects, besides MC-deficiency, that must also be considered while interpreting experimental findings. For example, it has been reported that C57BL/6 background Wsh mice exhibit enlarged and abnormal spleens, featuring increased megakaryocyte and myeloid populations [395]. In the bone marrow of Wsh mice, CD11b and Gr-1 (Ly6G)-expressing populations are also increased, including a marked expansion of circulating neutrophils [395]. Although the Wsh model provides a valuable starting point for evaluating the role of MC in response to FM *in vivo*, it is necessary to confirm that these findings are not significantly confounded by any associated immune defects. One strategy that is commonly employed in Wsh models is to reconstitute the local tissue site with primary MC, such as BMMC [394]. Reconstitution of Wsh mice with wildtype BMMC prior to FM injection would be important to confirm that the observed effects on pathology and recall responses are attributable to local MC.

4.4.3 MC in liver transplantation

In our MC-deficient model, we observed abrogated immune cell mobilization in response to FM. Both the early phase of hepatic IR and acute graft rejection are

characterized by immune cells trafficking to the injured tissue site and liver damage (Figure 1). The role of MC in liver IR is less investigated than other innate immune cells due to the lack of MC in the mouse liver. Future directions would be to further determine the role of MC in human liver transplantation by additional analyses. A first approach could be to examine levels of a MC degranulation associated proteases, such as tryptase, in liver transplant patient's plasma at various stages of transplantation and disease to explore if MC degranulation occurs after clinical liver transplant. Secondly, non-tolerant and tolerant liver biopsies could be used to check the number of MC in rejected or tolerant livers, or by using a rat liver transplant model with and without additional FM administration to explore the role of MC in liver transplantation.

Furthermore, acute lung injury is one of the most severe complications after orthotopic liver transplantation and a previous study found that MC stabilization could alleviate acute lung injury after orthotopic autologous liver transplantation [396]. Our study may provide a link between acute lung injury after liver transplantation to inflammation triggered by MC activation. Future experiments to assess inflammatory mediators in acute lung injury induced by FM including the role of MC mediators and FPR1 may provide further insights on acute lung injury after liver transplantation.

4.4.4 The role of FM in adaptive immunity

In our *in vivo* FM injection model, we found significantly enhanced DC recruitment. Given that the liver contains large amounts of hepatic resident DC and DC trafficking is a general feature in liver injury [397]. Future experiments could include assessing the function of DC in response to FM as well as assessing their ability to present antigen and invoke T cell responses in this context.

This study focused on innate and acute inflammatory responses triggered by extracellular mitochondria. However, it is likely that longer term unresolved chronic inflammatory responses can also result from such immune impacts and inflammatory mediator production. Indeed, the generation of antibodies directly against mitochondrial components may occur in liver transplant patients, chronic rheumatic diseases in which mitochondria are constantly liberated in synovial fluid, and in patients repeatedly transfused with extracellular mitochondria for the treatment of mitochondrial diseases [398, 399].

Interestingly, cardiolipin, a phospholipid uniquely expressed by mitochondria and bacteria, may be also highly antigenic, which provides an potential explanation for the prevalence of anti-cardiolipin in liver transplantation [400-404], different types of liver diseases [405-409], heart infarction [410-413] and rheumatic diseases [414]. Further investigations will be necessary to determine the extent of which mitochondria and MC contribute in these processes.

4.5 Conclusion

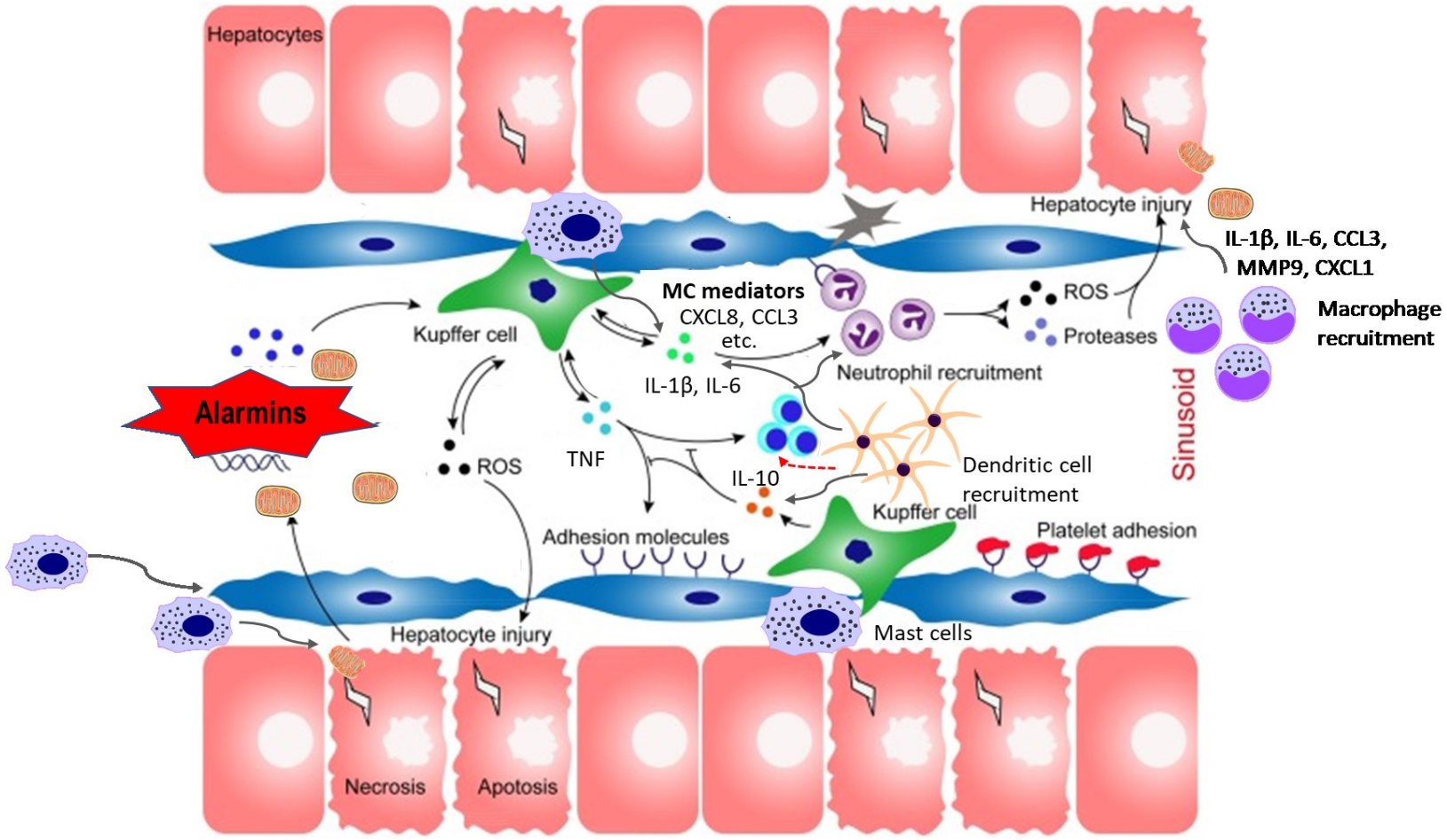
The growing body of scientific literature in the liver IR field, including *in vitro* observations, animal models of hepatic IR, and clinical data from human primary MC and human liver transplant databases, has significantly advanced our understanding of mitochondrial alarmin release during liver injury and pathogenesis of hepatic IRI (Figure 42). Through the presented work, we have elucidated a novel and significant role for innate immune cells, including MC and macrophages, in response to FM, and further clarified the impact of FPR1 mediated pathways. These studies have expanded our understanding of liver IRI immunity and may have implications for other organ ischemia injuries or sterile

injury with associated mitochondria release. Overall, these studies have provided new information concerning the mechanisms of inflammatory cytokine secretion and identified novel stimuli for the selective induction or production of critical MC and macrophage cytokines and chemokines. Although many further studies remain to be done, it is hoped that these findings will contribute to our future understanding of the role and regulation of the innate cells, such as MC and macrophages, in organs transplantation and related disease settings.

Figure 42. The role of FM as alarmins in liver injury.

Endogenous alarmins, such as FM, activated hepatic resident immune cells, such as MC and KC through FPR1 or TLRs to release inflammatory mediators. The alarmins facilitate local innate immune activation resulting in circulating neutrophils, DC, macrophages and MC migrating to the injured liver, which will further produce inflammatory cytokines leading to hepatocytes damage and degradation of the tissues. The pro-inflammatory cytokines also create a positive feedback loop that increases activity of KC and format inflammatory cascade reaction. Meanwhile, IL-10 produced from hepatic resident immune cells, such as KC, DC and MC counter-regulates the sustained pro-inflammatory activation. Adapted from [320].

Figure 42


Hepatocytes
Hepatocyte injury
IL-1β, IL-6, CCL3,
MMP9, CXCL1
Macrophage
recruitment
MC mediators
CXCL8, CCL3
etc.
ROS
Proteases
Kupffer cell
IL-1β, IL-6
Neutrophil recruitment
Alarmins
ROS
TNF
IL-10
Dendritic cell
recruitment
Sinusoid
Kupffer cell
Platelet adhesion
Adhesion molecules
Hepatocyte injury
Mast cells
Necrosis
Apotosis

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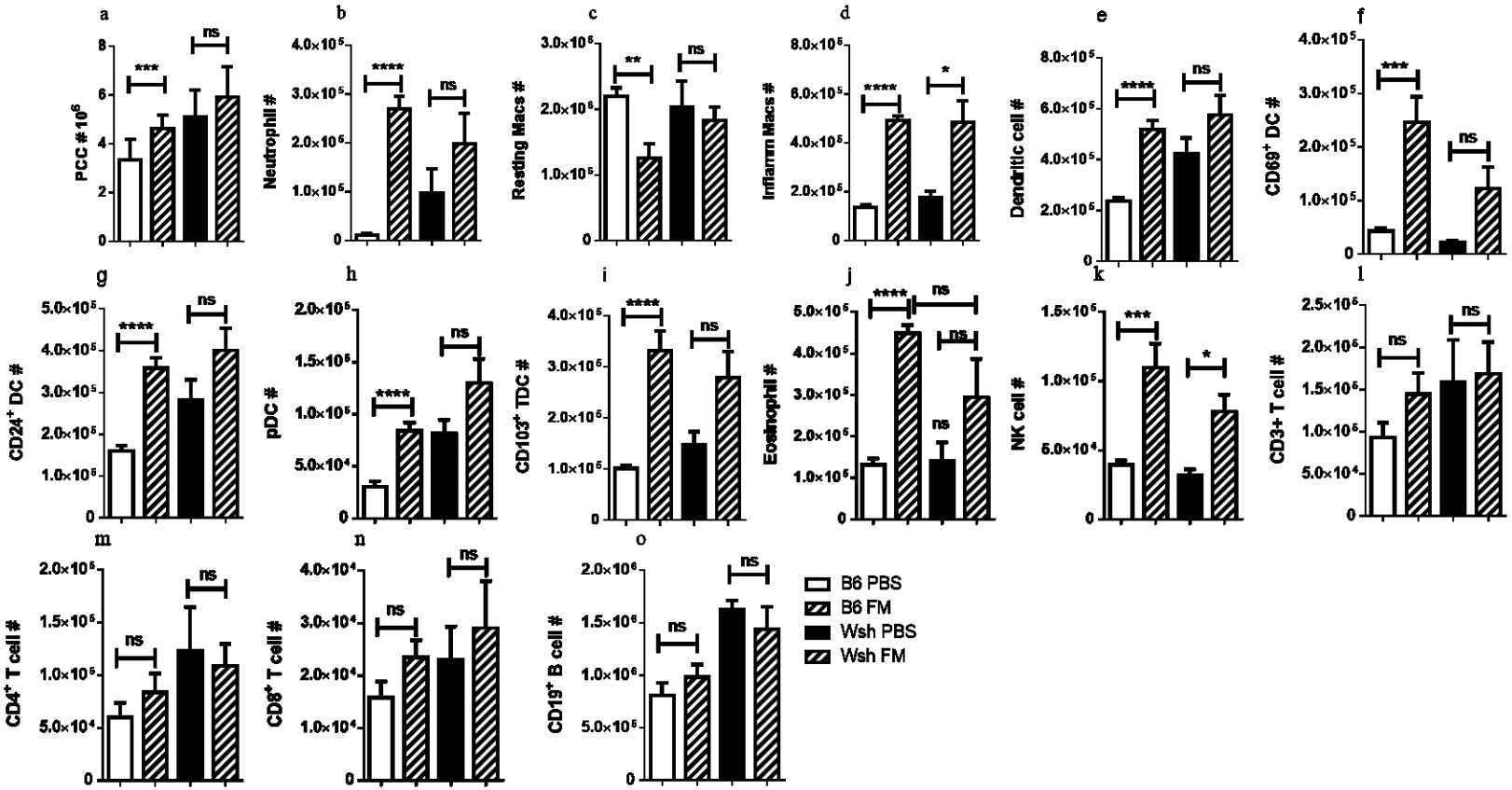
APPENDIX

Appendix 1. Comparison of FM induced peritoneal inflammation between C57BL/6 (B6) wildtype mice and C57BL/6-Kit$^{W\text{-}sh}$ (Wsh) mast cell deficient mice.

Wsh mice fail in FM induced myeloid cell recruitment. B6 and Wsh mice were injected i.p. with PBS or free mitochondria (FM, 5mg/kg) (equal to the mitochondria released by a 2.5% liver injury). After 16 hour the peritoneal contents were harvested by lavage, (a) the total peritoneal cavity cells (PCC) were counted, and the percentage of leukocytes in each samples were identified by flow cytometric analysis, cell number of (b)Neutrophils , (c) Resting macrophages, (d) Inflammatory macrophages, (e) Dendritic cells (DC), (f) CD69^{+} DC, (g)CD24^{+} DC Tolerogenic dendritic cells (TDC), (h) Plasmacytoid dendritic cells (pDC), (i) Tolerogenic dendritic cells (TDC), (j) Eosinophils, (k) Natural killer cells (NK), (l) CD3^{+} T cells, (m) CD4^{+} T helper cells, (n) CD8+ Cytotoxic T cells, and (o) B cells. The graphs shown are the mean±SEM, n=5- 11. *, $P< 0.05$; **, $P<0.01$; ****, $P< 0.0001$.

Appendix 1


a
PCC # 10^6
b
Neutrophil #
c
Resting Macs #
d
Inflamm Macs #
e
Dendritic cell #
f
CD69+ DC #
g
CD24+ DC #
h
pDC #
i
CD103+ TDC #
j
Eosinophil #
k
NK cell #
l
CD3+ T cell #
m
CD4+ T cell #
n
CD8+ T cell #
o
CD19+ B cell #
B6 PBS
B6 FM
Wsh PBS
Wsh FM