Persistent Hypermetabolism after Severe Burn Injury: Effects of Hepatic Stress and Regeneration

Li Diao

Doctor of Philosophy

Institute of Medical Science, University of Toronto

2019

Abstract

Burn injury represents one of the most severe forms of trauma. Persistent hypermetabolism and inflammatory response are common in major burned patients, contributing to morbidity and mortality. The underlying mechanisms are largely unknown and therefore novel and effective treatments are lacking. Liver is the fundamental mediator of post-burn immunologic and metabolic derangement and significant hepatomegaly is universally present and associated with the persistent hypermetabolism and inflammatory response in severely burned patients. We sought to understand if such a hepatomegaly is the consequence of 1) increased hepatic fat infiltration due to intensified lipolysis in white adipose tissue (WAT) and inter-organ crosstalk between liver and WAT, or 2) aberrant liver regeneration induced by stress response and liver damage which carries on hypermetabolic and pro-inflammatory signaling, contributing to persistent hypermetabolism and inflammatory response after major burn injury.
Rodent models of burn plus LPS administration, high fat diet (HFD) plus burn, and 30% TBSA burn of Sox9-cre/ERT2:ROSA26-EYFP mice were used for the studies.

In the rat model of burn plus LPS, we demonstrated 1) increased ER stress, inflammasome activation, apoptosis and lipolysis in WAT, contributing to liver steatosis (Chapter 2); 2) hepatic ER stress and inflammasome activation, contributing to liver damage and organ dysfunction (Chapter 3). In the mouse model of HFD plus burn, we showed that hepatic fat infiltration and metaflammation augment the liver damage and metabolic dysfunction post-burn (Chapter 4). We lineage-traced the facultative liver progenitor cells after burn injury and demonstrated that liver regeneration by this group of cells peaked around 2 weeks post-burn. Significant activation of multiple inflammatory and metabolic signaling pathways was indicated by transcriptomic analysis and verified by further analysis in the liver stem cells and their progeny post-burn as compared with both sham and self-renewal mature hepatocytes. Concomitant down-regulation of LXR signaling in the liver stem cells post-burn implicated the therapeutic potential of LXR agonist in ameliorating pro-inflammatory response and restoring lipid homeostasis after major burn injury (Chapter 5).

In conclusion, severe burn injury leads to hepatic stress response, liver damage and steatosis, stimulating liver regeneration from facultative stem cells which contributes to persistent hypermetabolism and pro-inflammatory response.
Acknowledgement

This will be my 2nd PhD degree (also most likely the last one) in my life and I have the opportunity to compare the learning experience between the young and the senior. I can tell the increased difficulty of a senior student in learning new things. However, coming back to school after 2 decades as a busy surgeon taking care of critically injured burn patients, it has always been very delightful to work on better understanding of the pathology of severe trauma patients and to appreciate the great potential of the advancement of modern biomedical science in improving the quality of the medical care and thus the quality of life of severely injured patients.

Upon the completion of the current PhD study, I would like to express my sincere thanks to my supervisor, Dr. Marc G Jeschke, for his mentorship, patience, and encouragement. It would be impossible for me to have this marvelous learning experience if without his many years of kindly support. Of equal importance is his role model of academic excellence, which, to me, is the perfect mixture of rigorous German and open-minded American. I will always remember: “…Mike, your research needs to be hypothesis driven!” I would also like to thank the members of my program advisory committee: Dr. Avery Nathens, Dr. Sandro Rizoli, and Dr. Ori Rotstein, for their support and suggestions that have been extremely helpful and added much value to my research. As what the Chinese sage Confucius said: “how happy I am, when meeting friends from far away”, I came a long way from the other side of the earth and also from a different cultural background to meet them and cherish the elegance and glamour of surgery and surgical research. I extend my special gratitude to Dr Saeid Amini-Nik for his generous help and guidance in detail in scientific research, from how to choose the proper mouse strain for animal experiments to the recommendation of science symposiums like Gordon and Keystone. As the participant of the Collaborative Program of Resuscitation Science, I would also express my thanks to Dr
Laurie Morrison who, together with Dr Rizoli and Dr Rotstein, hosted the program and provided enjoyable experience of group study for graduate students.

In the past 6 years, I spent most of the time in the Jeschke lab and I treasure all the happy memories with previous and current members of the lab. I have been learning a lot from everybody. As a Buddhist, I also benefited from the few hostile peers on how to keep calm, be humble, and be flexible.

I want to express my gratitude to all the members of the IMS office for their continuous care and support in my PhD study. Special thanks to Dr Mingyao Liu for his encouragement, inspiration and support all the way during my PhD study.

I feel so lucky to have many good friends around me and to be constantly blessed by them for the success of the scientific research in the past few years. Some friends are physically around as the family of Jed, Fiona and Anqi; Ma Bing and Michael; the family of Angus, Sophia and Shania; the family of Andy, Jenny and Lingling. Some are distant and I still clearly feel their warmth of care and support: Guocheng, Chen Hui, Helen, and their families.

I want to express my heartiest thanks to my parents, my parents-in-law, my brother and his family, my brothers-in-law and their families for their keen expectation and good wishes for my academic success. My gratitude goes to my parents and my parents-in-law for their altruistic support and I feel so blessed to see that they are happy and healthy and I wish them all the best for happy longevity. I appreciate my brother’s support in every aspect as we immigrate to Canada and I think a great deal of the industrious family of Rock, Jenny and Changchang and wish them good luck for future success.

I dedicate my thesis to my wife Alina and my son Joseph. It is their love and support that accompany me in our new life in Canada. I hope that this thesis may signify the future success of Alina together with our joint endeavor and motivate Joseph for his academic excellence.
Table of Contents

Abstract........................................................................................................................................................................................................... ii

Acknowledgements........................................................................................................................................................................ iv

Table of Contents........................................................................................................................................................................ vi

List of Tables................................................................................................................................................................................................ xi

List of Figures................................................................................................................................................................................ xii

List of Abbreviations......................................................................................................................................................................... xv

List of Publications (PhD study period)............................................................................................................................................ xviii

Prologue...................................................................................................................................................................................................... 1

Chapter 1 Introduction ................................................................................................................................................................... 3

1.1 Persistent pro-inflammatory response and hypermetabolism in major burned patients: liver as the mediator and the functional hub.......................................................................................................................... 3

1.2 Cellular stress response: the cellular basis of post-burn pathology.......................................................................................................................... 11

1.2.1 Historical perspectives.................................................................................................................................................................. 12

1.2.2 Heat shock responses (HSR)...................................................................................................................................................... 15

1.2.3 ER stress and UPR..................................................................................................................................................................... 17

1.2.4 Mitochondrial stress response and mitochondrial UPR (UPR\textsuperscript{mt})................................................................................. 20

1.2.5 Integrated stress response (ISR) determines cell function and cell fate and its Implication in the pathophysiology of critical illness................................................................................................. 24

1.2.5.1 ISR in hypoxia and ischemia and reperfusion injury......................................................................................................... 24

1.2.5.2 ISR upon infection and inflammation................................................................................................................................. 26

1.2.5.3 ISR in cell death and tissue and organ damage.................................................................................................................. 30

1.2.6 Summary.................................................................................................................................................................................. 32

1.3 Hepatic immunometabolic disorder, liver damage and regeneration after severe trauma injury.......................................................................................................................................................................... 33

1.3.1 Immunometabolism and hepatic inflammasome activation under stress conditions........ 33

1.3.2 Liver regeneration under profound stress condition and severe liver damage................. 38

1.4 Research problem, rationale and working hypotheses.................................................................................................................. 42
Chapter 2  Increased lipolysis in WAT and its contribution to hepatic fat infiltration .......................... 47

2.1 Introduction ........................................................................................................................................ 47

2.2 Materials and Methods ...................................................................................................................... 49

2.2.1 Animal model .................................................................................................................................. 49

2.2.2 Cell culture ....................................................................................................................................... 50

2.2.3 Plasma and tissue collection .......................................................................................................... 50

2.2.4 Gene expression analysis ............................................................................................................... 51

2.2.5 Western blotting ............................................................................................................................ 51

2.2.6 Immunofluorescent multi-channel staining of WAT ...................................................................... 52

2.2.7 H&E, Oil Red O (ORO), IHC and TUNEL staining of tissue sections ............................................. 53

2.2.8 Determination of FFA, glycerol and triglyceride levels in blood .................................................. 53

2.2.9 Statistical analysis .......................................................................................................................... 53

2.3 Results ................................................................................................................................................ 54

2.3.1 Burn and LPS induce significant catabolism and hepatic fat infiltration ...................................... 54

2.3.2 Increased WAT lipolysis in the 2-hit rat model of burn plus LPS .................................................. 56

2.3.3 Increased lipolysis in WAT after burn plus LPS is associated with reduced AMPK signaling ........ 58

2.3.4 Burn plus LPS increases adipocyte apoptosis ................................................................................. 61

2.3.5 Burn plus LPS synergistically induce apoptosis in WAT ................................................................ 64

2.3.6 Increased macrophage infiltration and inflammasome activation correlate with apoptosis in WAT ......................................................................................................................................................... 67

2.4 Discussion .......................................................................................................................................... 70

Chapter 3  Hepatic ER stress, inflammasome activation, liver dysfunction and damage ........................ 78

3.1 Introduction ......................................................................................................................................... 78

3.2 Materials and Methods ...................................................................................................................... 81

3.2.1 Animal model .................................................................................................................................. 81
3.2.2 Plasma and tissue collection ........................................................................................................ 82
3.2.3 Real-time quantitative RT-PCR ..................................................................................................... 82
3.2.4 Western blotting .............................................................................................................................. 84
3.2.5 Blood glucose level, plasma assay and IHC analysis for liver damage assessment ............. 84
3.2.6 Statistical analysis .......................................................................................................................... 85
3.3 Results................................................................................................................................................. 86
  3.3.1 The two-hit of burn injury with LPS injection induces liver damage .......................................... 86
  3.3.2 Burn plus LPS injection augment hepatic NLRP3 inflammasome activation ....................... 88
  3.3.3 Burn and LPS injection induce hepatic ER stress ..................................................................... 89
  3.3.4 Burn induces hypermetabolism .................................................................................................... 90
  3.3.5 Unlike burn which down-regulates SIRT1, LPS inhibits PKA C/AMPKα ................................... 92
3.4 Discussion........................................................................................................................................... 95

Chapter 4 Hepatic fat infiltration and liver damage ............................................................................. 102
  4.1 Introduction........................................................................................................................................ 102
  4.2 Materials and Methods....................................................................................................................... 103
    4.2.1 Animal model.............................................................................................................................. 103
    4.2.2 Plasma and tissue collection..................................................................................................... 104
    4.2.3 Western blotting......................................................................................................................... 104
    4.2.4 In-gel mitochondrial ETC activity assays.................................................................................. 105
    4.2.5 Immunofluorescent multi-channel staining of liver................................................................. 105
    4.2.6 H&E staining and TEM of tissue sections............................................................................... 105
    4.2.7 Determination of FFA, glycerol and triglyceride levels in blood........................................... 105
    4.2.8 Statistical analysis...................................................................................................................... 105
  4.3 Results................................................................................................................................................. 107
    4.3.1 HFD and burn lead to hepatic fat infiltration and increased lipolysis................................. 107
    4.3.2 De novo lipogenesis is not activated in HFD mice after thermal injury............................. 111
    4.3.3 Decreased hepatic lipid β-oxidation and attenuated mitochondrial ETC
function associate with hepatic fat infiltration............................................................... 113

4.3.4 Perturbed inter-organelle Ca2+ homeostasis correlates with decreased
ER-mitochondrial contact......................................................................................... 116

4.3.5 Augmented hepatic ER stress, inflammasome activation and aggravated
cell damage in HFD mice after thermal injury..................................................... 120

4.4 Discussion.......................................................................................................... 125

Chapter 5  Stress induces periportal ductal progenitor cells proliferation, contributing to
prolonged pro-inflammatory response and hypermetabolism .................................. 129

5.1 Introduction........................................................................................................ 129

5.2 Materials and Methods.................................................................................... 133

5.2.1 Animal model................................................................................................ 133

5.2.2 Liver tissue collection and digestion.............................................................. 134

5.2.3 Reagents and antibodies............................................................................. 135

5.2.4 Western blotting.......................................................................................... 135

5.2.5 Cell staining and flow cytometry................................................................. 135

5.2.6 Immunofluorescent multi-channel staining of liver........................................ 136

5.2.7 Microarray transcriptomic analysis............................................................... 136

5.2.8 Statistical analysis....................................................................................... 137

5.3 Results.............................................................................................................. 138

5.3.1 The proliferation of PDPCs increases, contributing to hepatomegaly after major
burn injury.............................................................................................................. 138

5.3.2 The hepatic stress response correlates with the increased proliferation of
PDPCs after major burn injury............................................................................. 143

5.3.3 Increased proliferation of PDPC-derived hepatocytes contributes to persistent
pro-inflammation and hypermetabolism after major burn injury.......................... 146

5.4 Discussion.......................................................................................................... 155

Chapter 6  Thesis summary and future directions....................................................... 162

6.1 General discussion............................................................................................. 162

6.1.1 Rodent animal models for translational research......................................... 163
List of Tables

Table 3.1 Primers sequences for qRT-PCR........................................................................................................ 83
Table 5.1 Primers for genotyping................................................................................................................. 133
Table 5.2 Microarray samples..................................................................................................................... 146
Table 5.3 Comparison of the changes in canonical signaling pathways in EYFP+  
cells in mice of Sham versus PBD7 group................................................................................................. 147
Table 5.4 Comparison of the changes in canonical signaling pathways in EYFP+  
versus EYFP- cells in mice of PBD7 group................................................................................................. 148
Table 6.1 Difference between the immunometabolic disorders after severe  
trauma and metaflammation..................................................................................................................... 167
List of Figures

Figure 1.1 Specific aims of the study........................................................................................................................................... 46

Figure 2.1 Burn and LPS induced catabolism and increased liver fat content................................................................. 55

Figure 2.2 Decreased adipocyte cell size and perilipin content in WAT of rats subjected to burn plus LPS............................... 57

Figure 2.3 Burn plus LPS do not directly activate HSL and MAPK lipolysis pathway...................................................... 59

Figure 2.4 Burn and LPS increased lipolysis by inhibiting AMPK signaling in WAT......................................................... 60

Figure 2.5 Burn plus LPS promoted apoptosis in WAT: TUNEL staining ........................................................................ 62

Figure 2.6 Burn plus LPS promoted apoptosis in WAT: evidence of pro-apoptotic signaling........................................ 63

Figure 2.7 Burn plus LPS increased ER stress which correlated with apoptosis in WAT.................................................... 65

Figure 2.8 Activation of pro-apoptotic signaling correlates with ER stress in adipose tissue in burn plus LPS in rats......... 66

Figure 2.9 Burn and LPS stimulated macrophage infiltration and inflammasome activation which correlated with apoptosis in WAT................................................................. 68

Figure 2.10 Activation of pro-apoptotic signaling correlates with macrophage Infiltration in adipose tissue in burn plus LPS in rats........................................................................ 69

Figure 2.11 No significant changes are detected in serum level of free fatty acid (A), glycerol (B) or triglyceride (C) among different treatment groups........................................ 73

Figure 2.12 Increased WAT lipolysis and its contribution to immunological and metabolic impairment in the 2-hit model of burn plus LPS........................................................................ 77

Figure 3.1 Enzymatic indicators and histological evidence of liver damage, weight loss and changes in blood glucose level in the two-hit rat model of burn and sepsis.................. 87

Figure 3.2 Two-hit models of burn plus LPS injection augments inflammasome formation in rat liver.......................... 88

Figure 3.3 Burn and LPS induced hepatic ER stress........................................................................................................... 89

Figure 3.4 Burn and LPS induced changes in gene expression of metabolic modulators in liver........................................ 91

Figure 3.5 Burn and LPS induced changes in PGC 1α in liver............................................................................................ 93

Figure 3.6 LPS reduced PGC-1α by inhibiting its upstream regulators........................................................................... 94

Figure 3.7 Hepatic ER stress and NLRP3 inflammasome activation exacerbate hepatic metabolic dysfunction and liver damage in the 2-hit rat model of burn plus LPS........... 101
Figure 4.1 16 weeks of HFD establishes obese mice with insulin resistance................................................................. 108

Figure 4.2 Augmented hepatic fat infiltration, increased lipolysis, and circulating FFA in obese mice after thermal injury .......................................................................................................................... 110

Figure 4.3 Repression of de novo lipogenesis in HFD mice after thermal injury................................................................. 112

Figure 4.4 Impaired hepatic lipid oxidation and mitochondrial electron transport chain (ETC) activities in the obese mice after thermal injury...................................................................................................... 115

Figure 4.5 Mitochondrial metabolic dysfunction is correlated with the perturbed inter-organelle Ca^{2+} homeostasis and mitochondrial dynamics in the liver of obese mice after thermal injury......................... 118

Figure 4.6 The decrease of hepatic ER-mitochondrial contact and mitochondrial structural changes after burn injury .................................................................................................................. 119

Figure 4.7 Augmented hepatic ER stress in HFD burned mice.............................................................................................. 121

Figure 4.8 TUNEL staining of liver tissue demonstrated increased liver cell apoptosis in HFD burned mice........... 122

Figure 4.9 NLRP3 inflammasome activation, enhanced pro-apoptotic signaling and DNA damage in HFD burned mice........................................................................................................................................ 124

Figure 4.10 Hepatic fat infiltration is attributable to the vicious cycle of ER stress, mitochondrial dysregulation and cell damage in HFD burned mice.................................................................................. 128

Figure 5.1 Different patterns of the expression of the key enzymes of lipid metabolism between the hepatocytes around portal triads and central venule systems................................................................. 131

Figure 5.2 Increased proliferation of the PDPCs contributes to hepatomegaly after thermal injury........................... 140

Figure 5.3 Increased proliferation of the PDPCs is around portal venule after thermal injury........................................... 141

Figure 5s.1 Optimization of the tamoxifen treatment protocol: dosage and route of administration.......................................................................................................................................................... 142

Figure 5s.2 The changes in body weight in mice before and after burn injury................................................................. 142

Figure 5.4 Hepatic cellular stress response after thermal injury.......................................................................................... 144

Figure 5.5 Hepatic cellular stress response correlates with PDPCs proliferation after thermal injury......................... 145

Figure 5.6 Up-regulation of the acute phase response (A), p38 MAPK (B), and IL-6 (C) signaling pathways in PDPCs after thermal injury...................................................................................................... 149

Figure 5.7 On PBD7, acute phase response (A), p38 MAPK (B), and IL-6 (C) signaling pathways are more activated in PDPCs as compared with that in mature hepatocytes................................................ 150

Figure 5.8 LXR/RXR signaling pathway is significantly down-regulated in PDPCs ............................................................................. 151
Figure 5.9 Up-regulated hepatic acute phase response and p38 MAPK signaling followed the decrease of LXRα expression and correlated with increased lipid oxidation and cell damage in the liver after thermal injury ................................................................. 154

Figure 5.10 Aberrant liver regeneration contributes to persistent pro-inflammatory response and hypermetabolism after major burn injury ........................................................................................................ 161

Figure 6.1 Hepatic stress response, liver damage and regeneration contribute to persistent pro-inflammatory response and hypermetabolism after major burn injury ............................................. 175
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>αMSH</td>
<td>α-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>Arg 1</td>
<td>arginase 1</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signaling kinase 1</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>ATGL</td>
<td>desnutrin/adipose triglyceride lipase</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>C/EBPs</td>
<td>CCAAT/enhancer-binding-proteins</td>
</tr>
<tr>
<td>CGI-58</td>
<td>comparative gene identification-58</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/Enhancer-Binding Protein Homologous Protein</td>
</tr>
<tr>
<td>cLDs</td>
<td>cytoplasmic lipid droplets</td>
</tr>
<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
</tr>
<tr>
<td>CRTC2</td>
<td>CREB-regulated transcription coactivator 2</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2α</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ESLD</td>
<td>end-stage liver disease</td>
</tr>
<tr>
<td>FAO</td>
<td>fatty acid oxidation</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>HG</td>
<td>high glucose</td>
</tr>
<tr>
<td>HMGB1</td>
<td>high mobility group box protein 1</td>
</tr>
<tr>
<td>HOP</td>
<td>HSP-organizing protein</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic–pituitary–adrenal</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
</tr>
<tr>
<td>HSF1</td>
<td>heat shock factor 1</td>
</tr>
<tr>
<td>HSL</td>
<td>hormonal-sensitive lipase</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSR</td>
<td>heat shock response</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescent</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor-1</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemical</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IMS</td>
<td>intermembrane space</td>
</tr>
</tbody>
</table>
iNOS   inducible nitric-oxide synthase
IP3R   inositol 1,4,5-triphosphate receptor
IPGTT  intraperitoneal glucose tolerance test
IR     insulin resistance
IRE1   inositol-requiring kinase 1
IRF    interferon-regulatory factor
ISR    integrated stress response
JNK    c-Jun N-terminal kinase
LAL    lysosomal acid lipase
LDL    low-density lipoproteins
LFD    low fat diet
LPS    lipopolysaccharides
LXR    liver X receptor
MAG    2-monoacylglycerol
MAPK   mitogen-activated protein kinase
MCP-1  monocyte chemoattractant protein-1
M-CSF  macrophage colony-stimulating factor
MCSR   mitochondria to cytosol stress response
MFN2   mitofusin 2
MGL    monoacylglycerol lipase
MLKL   mixed lineage kinase like
mPOS   mitochondrial precursor over-accumulation stress
MPT    mitochondrial permeability transition
mTOR   mechanistic target of rapamycin
MTS    mitochondrial targeting sequences
NAFLD  nonalcoholic fatty liver disease
NASH   nonalcoholic steatohepatitis
NF-κB  nuclear factor-κB
NLR    NOD-like receptor
NLRP3  NOD-like receptor, pyrin domain containing 3
OPA1   Dynamin-like 120 kDa protein, mitochondrial
ORO    oil red O
OXPHOS Oxidative phosphorylation
PAMP   pathogen-associated molecular pattern
PARP   poly ADP ribose polymerase
PBA    phenylbutyrate
PBD    post-burn day
PBS    phosphate-buffered saline
PCNA   proliferating cell nuclear antigen
PDI    Protein disulfide isomerase
PDPC   periportal ductal progenitor cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PERK</td>
<td>pancreatic ER eIF2a kinase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma coactivator 1-α</td>
</tr>
<tr>
<td>PHIR</td>
<td>Persistent hypermetabolism and inflammatory responses</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PKA C</td>
<td>Protein kinase A catalyst unit</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>REE</td>
<td>resting energy expenditure</td>
</tr>
<tr>
<td>RIDD</td>
<td>regulated IRE1-dependent decay of mRNA</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RIP</td>
<td>regulated intramembrane proteolysis</td>
</tr>
<tr>
<td>RIPK</td>
<td>receptor interacting protein kinase</td>
</tr>
<tr>
<td>RLR</td>
<td>RNA cytosolic helicases of the RIG-1-like receptors family</td>
</tr>
<tr>
<td>Rnase</td>
<td>endoribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SIK1</td>
<td>salt inducible kinase 1</td>
</tr>
<tr>
<td>Sox9</td>
<td>Sry-related HMG box 9</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor beta-activated kinase 1</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TBSA</td>
<td>total body surface area</td>
</tr>
<tr>
<td>TFE3</td>
<td>transcription factor E3</td>
</tr>
<tr>
<td>TFEB</td>
<td>transcription factor EB</td>
</tr>
<tr>
<td>TG</td>
<td>triglycerides</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF2</td>
<td>tumor necrosis factor α receptor-associated factor 2</td>
</tr>
<tr>
<td>TRAP1</td>
<td>TNF Receptor-Associate Protein 1</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box-binding protein 1</td>
</tr>
</tbody>
</table>
List of Publications (PhD study period)


In May 2012, right before entering into the PhD program, I read 3 published papers on the clinical study of the major burned patients, written by my PhD supervisor Dr Jeschke, in which he wrote:

“...it clearly demonstrated that burn induced metabolic and inflammatory changes persisted for 3 years after the injury” in a study in which “Nine-hundred seventy-seven severely burned children were included...” (Jeschke et al., 2011a). Such prolonged hypermetabolism and inflammation is linked with multiple post-burn comorbidities including increased risk for infection and sepsis and increases the mortality of the patients.

“... the change in serum triglycerides and free fatty acids, both of which are significantly increased through almost the entire acute hospital stay...A therapeutic approach to decrease lipolysis and fatty infiltration and reverse the acute phase response may thus improve morbidity and mortality” (Jeschke et al., 2008a).

Concomitant to the catabolism seen in multiple organs and systems including muscle, bone, and peripheral adipose tissue, there is a “massive hepatomegaly and hepatic fatty infiltration” observed in both “…burn victim at autopsy” as well as “…in 242 surviving burn patients” (Jeschke, 2009), depicting pivotal role of the liver in the post-burn pathophysiology in which hypermetabolism and inflammation are featured.

In the past 6 years, I took on the journey of biomedical research, investigating the nature of stress response and lipolysis in white adipose tissue (WAT), and seeking the
relationship between such lipolysis and liver fat infiltration and the impact of the latter on liver
dysfunction and damage.

In addition to the aforementioned stress induced cell damage and organ dysfunction,
considering the huge potential of liver regeneration after injury and the central role of liver as
the hub integrating the whole body regulation of metabolism and immunology, I also
hypothesized and sought to prove that profound stress response after burn injury stimulates
liver regeneration which is different to physiological renewal of hepatic parenchyma, and
contributes to the prolonged hypermetabolism and hyper-inflammatory state.
Chapter 1 Introduction

1.1 Persistent pro-inflammatory response and hypermetabolism in major burned patients: liver as the mediator and the functional hub

Our previous clinical studies clearly demonstrate the presence of prolonged pro-inflammatory and hypermetabolic responses that lead to hyper-dynamic circulation, increased body temperature, glycolysis, proteolysis, lipolysis and futile substrate cycling in patients with major burn over 30% total body surface area (TBSA) (Jeschke et al., 2008a; Jeschke et al., 2011a). Such a prolonged post-burn metabolic and inflammatory changes are accompanied with sustained increase in catecholamines and stress hormones, persistent elevation of resting energy expenditure (REE), increased expression of inflammatory cytokines such as TNF, IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), and persistent elevated levels of blood glucose and insulin depicting significant insulin resistance. The consequence of this persisting post-burn metabolic and inflammatory response is detrimental, contributing to impaired wound healing, increased incidence of wound and systemic infection, prolonged post-burn rehabilitation and even increased mortality. Indeed, in our recent prospective cohort study, we have shown that significantly higher serum levels of IL-6, IL-8, G-CSF, MCP-1, C-reactive protein, glucose, insulin, blood urea nitrogen, creatinine, and bilirubin predicted higher likelihood of mortality and non-survivors exhibited a vastly increased hypermetabolic response that was associated with increases in organ dysfunction and sepsis.
(Jeschke et al., 2014a). The research to elucidate the underlying mechanisms of aggravating pro-inflammatory response and hypermetabolism is thus warranted for effective treatment.

Considering that the liver is the functional hub integrating metabolic response, immunologic response, inflammatory response and acute phase response, we have long been emphasizing the importance of the liver in mediating the metabolic and inflammatory disorders post-burn (Jeschke, 2009; Jeschke et al., 2004).

Aberrant hepatic metabolic responses after major burn injury are manifested by the derangement of glucose metabolism, increased proteolytic signaling, and dysregulated lipid metabolism.

Hepatic glucose metabolism is regulated through diverse mechanisms. Hepatic glucose production is regulated by 1) the provision of substrates, such as glucose or glycerol; 2) allosteric control by metabolites, such as acetyl-CoA, glucose and glucose-6-phosphate; 3) the balance of hormones, including insulin, glucagon, catecholamine and corticosteroids; and 4) cellular redox state, which can be modified by treatment with metformin (Petersen et al., 2017). Stress mediators, such as catecholamine, glucocorticoid, glucagon, and dopamine, stimulate lipolysis in adipose tissue and proteolysis in skeletal muscle thus increase the substrates of gluconeogenesis; catecholamine and glucagon can also mediate enhancement of hepatic glycogenolysis, all contributing to hyperglycemia post-burn. Pro-inflammatory cytokines, including TNF, IL-6 and MCP-1, directly act on the insulin signal transduction pathway through modification of the signaling properties of insulin receptor substrates, contributing to post-burn hyperglycemia via hepatic insulin resistance (Mecott et al., 2010).
Increased proteolysis and muscle wasting are common pathology of major burned patients. Although this is out of the scope of the current PhD research project, we noticed that in recent years, in the area of the research of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), the concept of sarcopenia is called into attention which is defined as a progressive and generalized loss of skeletal muscle mass, strength, and function commonly seen in up to 60% of patients with end-stage liver disease (ESLD), depicting the importance of hepatic pathophysiology in the changes in the protein catabolism under critical illness (Bhanji et al., 2017). Interestingly, since mechanisms relating sarcopenia to NASH include IR, increased inflammation, myokines secreted by skeletal muscle, myostatin, adiponectin, vitamin D deficiency, and physical inactivity (Merli and Dasarathy, 2015), it is reasonable to speculate the interaction between sarcopenia and post-traumatic IR and pro-inflammatory state which are at least partly originated from liver pathology. Specifically, IR increases lipolysis in adipose tissue with the consequent release of free fatty acids (FFAs) to the liver and high levels of FFA inhibit the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis, contributing to muscle loss and decrease in muscle regeneration (Kalyani et al., 2014); enhanced fatty acid oxidation (FAO) in the liver leads to generation of oxygen free radicals, which causes lipid peroxidation and induces synthesis of pro-inflammatory cytokine such as tumor necrosis factor-α (TNFα) which not only leads to direct liver injury, but also stimulate protein catabolism, which results in loss of muscle mass and sarcopenia (Phillips and Leeuwenburgh, 2005).
Dysregulated hepatic lipid metabolism features hepatomegaly and hepatic fat infiltration which could be attributed to excessive delivery of fatty acids to the liver as a consequence of \( \beta \)-adrenergic mediated stimulation of lipolysis and a diminished effectiveness of insulin in suppressing lipolysis. It has also been suggested that decreased VLDL-triglyceride secretion is seen in burn patients and is unresponsive to increased hepatic triglyceride synthesis (Morio et al., 2002). Hepatomegaly and hepatic steatosis and dysfunction in severely burned rats are associated with increased mortality and that liver integrity and function are crucial for survival post-burn (Mittendorfer et al., 1998). In IL-6 knockout mice which developed cholestasis, steatosis, and hepatocellular injury upon cecal ligation and puncture (CLP), there is an aggravated hepatic dysfunction and increased mortality in sepsis (Deutschman et al., 2006). All these observations indicate that hepatomegaly and hepatic fat infiltration are detrimental to the outcome of major burn injury and infection.

In a recent review of the metabolic stress response to burn trauma (Porter et al., 2016), it has been corroborated that persistent pathophysiological stress response of adrenergic and inflammatory stress, hypermetabolism, metabolic dysfunction, and reduced lean body mass can be presented for up to and beyond 3 years after burn injury of more than 20% TBSA. It has also been clearly demonstrated that the activation of uncoupling protein 1 (UCP1) expression in functional brown and subcutaneous white adipose tissues upon persistent adrenergic stress post-burn contributes to increased energy expenditure and hypermetabolic response (Patsouris et al., 2015; Sidossis et al., 2015). Accordingly, it has been suggested that browning of white adipose tissue is causative to post-burn hypermetabolism and to inhibit or alleviate such
browning is proposed to be therapeutic to decrease hypermetabolism and improve clinical outcome (Abdullahi and Jeschke, 2017).

However, from the point of view of evolution, browning of the adipose tissue is one of the most important adaptive mechanisms of thermogenesis and it has been strongly implicated as protective and beneficial to the living organisms under different stress conditions. If such beneficial thermogenesis in mammals generally hold true, we might speculate another regulatory mechanism when taking into consideration the central insulin resistance of the liver: persistent and profound adrenergic stress signaling post-burn stimulates lipolysis in the adipose tissues and contributes to the increased hepatic lipid influx; hepatic fat infiltration contributes to increased hepatic glucose production and output; browning of the adipose tissue may thus be an adaptive and protective mechanism to neutralize the detrimental effect of such lipolysis and consequent hepatic lipotoxicity. Hence, it is important to further clarify the impact of browning of white adipose tissue in the post-burn pathology.

Regardless of the dispute and controversy in the significance of browning of the white adipose tissue in the pathophysiology after major burn injury, it is clear that attenuating lipolysis may decrease the hepatic lipid preload thus is beneficial to the restoration and maintenance of hepatic homeostasis.

There are three mechanistically cooperate principal pathways of intracellular lipolysis, which are neutral lipolysis of cytoplasmic lipid droplets (cLDs), acid lipolysis in lysosomes, and lipophagy (Zechner et al., 2017). The most common neutral lipolysis in adipose and non-adipose tissues initiates from triacylglycerol hydrolysis by adipose triglyceride lipase (ATGL) to form...
diacylglycerol and FFAs (Zimmermann et al., 2004). Hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) complete the process by consecutively hydrolyzing diacylglycerols into monoacylglycerols and FFAs and hydrolyzing monoacylglycerols into glycerol and FFAs (Vaughan et al., 1964). Endocrine regulation of neutral lipolysis is complex and involves numerous hormones, growth factors and adipokines that are linked to diverse signal transduction pathways. Catecholamines, glucagon, thyroid-stimulating hormone and melanocortins, natriuretic peptides, and pituitary growth hormone (somatotropin) are able to activate neutral lipolysis via the cAMP–PKA pathway in which a number of cLDs-associated proteins, including perilipin 1, HSL and comparative gene identification-58 (CGI-58), are phosphorylated whereas insulin and insulin-like growth factors as well as non-hormone inhibitors like lactate, adenosine, β-hydroxybutyrate and nicotinic acid (niacin), mTOR complex 1 (mTORC1), mTORC2 and AMPK are the inhibitors of lipolysis. Perilipin 1 phosphorylation at multiple residues leads to the release of CGI-58, which is then able to activate ATGL. Simultaneously, phosphorylated HSL translocate from the cytosol to cLDs. HSL regulation by enzyme phosphorylation is complex. Five distinct serine residues (Ser563, 565, 600, 659, 660) are phosphorylated by either activating kinases (PKA, PKG and extracellular-signal-regulated kinases (ERKs)) or inhibitory kinases (AMP-activated protein kinase (AMPK)), calcium/calmodulin-dependent protein kinase type II and glycogen synthase kinase 4), which respectively trigger or prevent HSL translocation and activation (Watt and Steinberg, 2008). However, the role of AMPK in the regulation of lipolysis is less well defined since AMPK is activated during fasting and exercise, when cellular AMP concentrations increase, but whether or not this induction contributes to the upregulation of lipolysis is still controversial (Ceddia,
The second principal pathway of intracellular lipolysis is acid lipolysis in lysosomes where triacylglycerol degradation is carried out by lysosomal acid lipase (LAL) owing to its optimal activity at the lysosomal pH of 4.5-5. This pathway was assumed to be mainly responsible for the degradation of exogenous plasma lipoprotein-associated lipids, including triacylglycerol. LAL is highly glycosylated and exists in various tissue-specific isoforms and it can be secreted from cells via the classical endoplasmic reticulum (ER)–Golgi secretory pathway and can subsequently re-enter cells and lysosomes by endocytosis. Since lysosomes are unable to store any degradation products, the catabolic machinery, including LAL, and lysosomal export mechanisms are constitutively active. Therefore, the regulation of acid lipolysis, and specifically LAL occurs predominantly at the gene transcription stage and FOXO1, transcription factor EB (TFEB), transcription factor E3 (TFE3), PPARα and its co-activator, PGC1α are among the many that promote the LAL transcription (Emanuel et al., 2014; Settembre et al., 2013). The third lipolysis pathway is lipophagy which relies on the same general mechanisms as macroautophagy involving more than 30 ATG-encoding genes. This is strongly induced by the major metabolic kinases mTORC1 and AMPK during lengthy fasting and the activity of these kinases depends on growth factor signaling, the cellular energy status (ATP: AMP ratio) and nutrient availability. Nutrient-mediated transcriptional regulation of hepatic autophagy also occurs through the nuclear receptors PPARα and the liver X receptors (Lamb et al., 2013; Lee et al., 2014).
Considering the above complex nature of the lipolysis and its importance in the development of the hepatic metabolic derangement, it warrants further investigation how the lipolysis is involved in the pathology of major burn injury.

It has become clear that hepatic immunologic and pro-inflammatory responses under stress conditions after severe trauma injury are closely related and synergistically regulated with the metabolic response, and such an interaction has been conceptualized as immunometabolic disorder which will be discussed in detail in the third section of this chapter.

Another important aspect of hepatic involvement of post-burn pathology is the activation of the acute phase response which is believed to represent a re-direction of the liver to fulfill immune functions, metabolic responses, coagulation, and wound healing processes (Jeschke et al., 2008a). It has been demonstrated that pro-inflammatory cytokines mediate the acute phase response and the signal transcription cascade includes various pro- and anti-inflammatory signal transcription factors such as c-jun/c-fos, nuclear factor-kappa B (NF-κB), CCAAT/enhancer-binding-proteins (C/EBPs), tyrosine phosphorylation and activation of intracellular tyrosine kinases (JAKs), latent cytoplasmic transcription factors, signal transducer and activator of transcription 1 (STAT1), STAT3, and STAT5, or mitogen-activated protein (Klein et al., 2003). The surge of acute phase response is concomitant with the down-regulation of the synthesis of the constitutive hepatic proteins. Accordingly, although the acute phase response could be beneficial to protect the body from further damage if all elements of the acute phase response coalesce in a balanced fashion, a prolonged increase in pro-inflammatory cytokines
and acute phase proteins has been shown to be indicative of a hyper-catabolic state, associated with an increased risk of sepsis, multi-organ failure, morbidity and mortality.

In summary, the correlation between the liver dysfunction/damage and inflammatory and metabolic disorders in major burn patients has been well-established, further mechanistic studies are warranted to investigate the interaction among hepatic stress response, liver dysfunction, and liver damage and to elucidate how such an interaction contributes to prolonged inflammatory and metabolic derangement.

1.2 Cellular stress response: the cellular basis of post-burn pathology

Burn injury represents one of the most severe forms of trauma in which pervasive perturbation of homeostasis occurs in almost all the organs and systems and lasts for a prolonged period of time. Such a perturbation of homeostasis leads to stress responses at the cellular level (Jeschke et al., 2012). While the cellular stress response is highly conserved throughout the evolution, the hierarchical difference among the species is still significant. In single cell organisms, the stress response solely aims at restoring homeostasis and thus pro-survival as what we refer to as “to be or not to be, that is the only question”; in organisms of multi-cellular and higher level when the benefit and risk of single cell death or survival should be taken into consideration for the general interest of the whole body, evolutionary pressure is
in favor of flexible thus multifaceted mechanisms of stress responses to ensure not merely the protection of the individual cell but also that under certain circumstances when the cell damage is inevitable, the pro-survival signaling can be quickly turned into pro-apoptotic one to facilitate apoptosis thus effectively contain the detrimental effect of the insult within the damaged cells for the best interest of the homeostasis and survival of unaffected cells and, ultimately, the living body as a whole.

How such flexible and multifaceted stress responses are initiated and regulated is of persevering interest of biological research for more than half of a century and a rich body of literature has been accumulated. It is necessary to summarize the development of our understanding toward the nature of the stress responses, especially in mammals and human, so as to elaborate how the cellular stress responses are triggered, regulated, and linked with other cell physiology at the subcellular level, and to speculate the pathological implications of such stress responses in the injuries and illnesses.

1.2.1 Historical perspectives

In 1962, Ferruccio Ritossa published his seminal paper of the heat shock response (HSR) in the larval of the Drosophila upon the raising of the incubating temperature (Ritossa, 1962). This is the milestone of the initiation of our understanding of cellular stress response which stands only one year after the discovery of mRNA (Brenner et al., 1961). 12 years later, Tissieres
et al. reported that the induction of such heat shock response coincided with the synthesis of the new proteins which were later named heat shock proteins (Tissieres et al., 1974).

In the following 10 more years, multiple heat shock (stress) proteins were identified and isolated, classified into different groups according to the molecular weight. Their genes were cloned, and they were gradually distinguished among each other for their functions in different cellular physiological or pathological processes (Lindquist, 1986). It came out that except for the increased temperature, different environmental changes can induce the increased gene expression of this large group of proteins and thus they are preferably termed stress proteins and, in most cases, they work as molecular chaperones which dynamically interact with the unfolded or mis-folded target proteins at their exposed hydrophobic patches, specific peptide sequences, or structural elements of the nonnative proteins thus facilitate the optimization of the efficient and correct folding of these substrate proteins to facilitate proper folding or stabilize the structure of the target proteins (Richter et al., 2010; Welch, 1992).

However, neither the mechanisms by which the cells recognize the adverse changes in the environment and increase the expression of certain stress proteins, nor the exact location of such stress proteins take effects was clearly defined until the publication of Kozutsumi’s work in 1988 (Kozutsumi et al., 1988). In this paper, not only was it clearly demonstrated that the increase in the unfolded or mis-folded proteins is the inducer of the expression of the stress proteins, but also that two stress proteins studied, namely glucose regulated protein 78 and 94 (GRP78 and GRP94), are located in the endoplasmic reticulum (ER) while most of the canonical heat shock proteins are cytosolic. This is the commencement of the research in ER stress which
brings the studies of stress response to the subcellular level. Indeed, considering that the ER is where the gene translation occurs and newly synthesized, nascent peptide chains fold to form stereo structures for functional proteins, it is not surprising that, despite being called ER stress proteins, these molecular chaperones play pivotal roles in maintaining physiological function of the cells (Bukau et al., 2006). Nevertheless, more attention has been called to study the unfolded protein response (UPR) and ER stress which proves to be involved in a wide spectrum of illness (Jeschke et al., 2012; Ozcan et al., 2004).

In parallel with the initiation of the studies of stress response in the ER, scientists also paid attention to the stress response in the mitochondria (Deshaies et al., 1988). However, since 1) the stress response of the mitochondria involves stoichiometry of mitochondrial- and nuclear-encoded proteins; 2) the mitochondria are double-membraned structures with dynamic and fluctuating transmembrane potential; 3) there are constant biochemical reactions of oxidative phosphorylation (OXPHOS) and reactive oxygen species (ROS) production; 4) mitochondria are highly dynamic organelles (under constant fission and fusion) and subject to cellular quality control mechanisms for degradation upon damage, the mitochondrial stress response and unfolded protein response are more complicated and it took much longer time to form a blueprint for it than that for the ER stress (Haynes and Ron, 2010).

Indeed, until very recently, with the better understanding of such a complicated mitochondrial stress responses, there has been an increased appreciation of the integration of the stress responses in the cytoplasm, ER, mitochondria, and nucleus which results in a cell-autonomous reprogramming in different pathological conditions (D'Amico et al., 2017; Ruan et
al., 2017; Schito and Rey, 2018; Sorrentino et al., 2017). Accordingly, in the foreseeable future, we are looking forward to more systematic elucidation of the cellular stress response which may shed lights on novel therapeutic interventions for more effective restoration and better maintenance of homeostasis when facing harmful insults of different origin.

1.2.2 Heat shock response (HSR)

The cytosolic HSR is the first line of the adaptive mechanisms toward the stressful conditions. However, this part is not within the scope of my current PhD research program. To maintain the inclusiveness of the literature review, I briefly summarize here the basic concept of the HSR. It is implicated that the structural changes of biomolecules in the cytosol happen in advance of the genetic regulation. Specifically, deleterious environmental changes exemplified as heat shock bring about intracellular changes including 1) reorganization of the cytoskeleton from stress fiber formation of actin filaments, aggregation of vimentin or other filament-forming proteins, to the collapse of intermediary, actin and tubulin networks (Toivola et al., 2010); 2) loss of correct localization of intracellular organelles such as fragmentation of Golgi system and ER, as well as decrease of the number of mitochondria and lysosomes (Welch and Suhan, 1985); 3) formation of nucleoli (Boulon et al., 2010) and stress granules (Buchan and Parker, 2009) containing incorrectly processed ribosomal RNAs, aggregating ribosomal proteins, non-translating mRNAs, translation initiation components, and other proteins affecting mRNA function; 4) changes in membrane morphology and the ratio of protein to lipids which result in
higher fluidity of the membranes and increased membrane permeability and consequently, drop in cytosolic pH and changes in ion homeostasis (Vigh et al., 2007).

Upon such perturbation of the homeostasis, HSR is triggered and mediated predominantly by the heat-shock factor (HSF) family of transcription factors to maintain proper protein-folding in the cytosol. Mechanistically, constitutive HSP70 and HSP90 bind to the trans-activating domain of HSF1, thus repressing its transcriptional activity under normal conditions. Following either heat shock or any other condition that perturbs protein folding within the cytosol, HSP70 and HSP90 preferentially interact with the accumulating unfolded proteins, thus releasing HSF1 and allowing it to translocate from the cytosol to the nucleus and bind as a homotrimer to heat shock elements (HSEs), the promoter consensus sequences that regulate the expression of heat shock genes, and transcriptionally activate the genes including HSP27, HSP70, HSP90 and proteasome subunits (Velichko et al., 2013). Among these, HSP27 disaggregates nuclear proteins, provides significant resistance from heat shock and oxidative stress, and plays a role in the repair and restoration of the cytoskeleton structures (Singh et al., 2017); HSP70 and HSP90 facilitate nascent and mis-folded protein folding, protein trafficking and subcellular sorting (Young et al., 2004); and ubiquitin tags the damaged or other targeted proteins for their degradation in the proteasome (Varshavsky, 2017).
1.2.3 **ER stress and UPR**

The emergence of the ER in the process of the evolution is concomitant with the evolutionary jump from prokaryotes to eukaryotes. The membranous structures of eukaryotes, including nuclear membrane, ER, Golgi complex, and mitochondria, compartmentalize the cell to multiple interacting yet separate units. This not only makes it possible that, within the cell of a much larger size, parallel and efficient biochemical reactions can be accurately modulated to optimize the cell function, but also renders the cell much higher complexity when facing the perturbation of the homeostasis. Since ER is where numerous secretory and structural proteins are synthesized, folded to form functional structure, and further modified for trafficking and quality control, molecular chaperones, such as GRP78/BiP, GRP94, and GRP170, play important roles in all these processes and thus keep a high expression level under physiological conditions (Schroder and Kaufman, 2005).

Furthermore, it has been clearly demonstrated that BiP, the ER resident HSP70 homologue, is binding with multiple ER transmembrane signaling molecules under physiological conditions and thus keeping these molecules in the inactivated states. Upon stress conditions, the ER protein synthesis increases and unfolded and mis-folded proteins accumulate. BiP preferentially bind to these unfolded and mis-folded proteins and release the binding with those transmembrane ER signaling molecules, including inositol-requiring kinase 1 (IRE1), pancreatic ER eIF2α kinase (PERK), and activating transcription factor 6 (ATF6), thus activating the three branches of ER UPR (UPRER) (Cao and Kaufman, 2012).
Mammalian IRE1 has two homologues: IRE1α and IRE1β. IRE1α is expressed ubiquitously and IRE1β is strictly expressed in the intestinal epithelial cells. The IRE1 has two cytosolic domains of a serine/threonine kinase domain and an endoribonuclease (RNase) domain, corresponding to two mechanisms of activation upon dimerization when released from BiP binding under stress conditions (Kimata et al., 2004). Firstly, phosphorylation of IRE1α in the cytosolic domain stimulates its interaction with tumor necrosis factor α receptor-associated factor 2 (TRAF2), an adaptor protein in the TNFα signaling pathway, which recruits IκB kinase (IKK) to phosphorylate and degrade IκB thus activates nuclear factor-κB (NF-κB) and its downstream inflammatory pathways (Tam et al., 2012). The IRE1α–TRAF2 complex also recruits apoptosis signaling kinase 1 (ASK1), which activates c-Jun N-terminal kinase (JNK) to stimulate pro-inflammatory response signaling by the AP1 transcription factor phosphorylation (Ron and Walter, 2007). IRE1α–JNK was also suggested to activate pro-apoptotic pathways and induce insulin resistance by phosphorylating insulin receptor substrate 1 and 2 in response to ER stress (Liang et al., 2015). Secondly, IRE1 dimerization activates luminal domain of RNase which initiates the splicing of X-box-binding protein 1 (XBP1) and degradation of a subset of mRNA to reduce protein synthesis to alleviate ER stress (regulated IRE1-dependent decay of mRNA, RIDD). Spliced XBP-1 is a potent transcription activator, inducing the expression of a wide range of genes that orchestrate ER protein folding, secretion, quality control and ER-associated degradation (ERAD), and activates phospholipid biosynthesis and ER expansion upon ER stress. It is thus implicated in a wide spectrum of biological processes, including differentiation, metabolism, inflammation, tumorigenesis and neurodegeneration (He et al., 2010).
PERK is activated upon releasing from BiP under stress conditions by oligomerization and trans-autophosphorylation. Activated PERK phosphorylates Ser51 of α subunit of eukaryotic translation initiation factor 2α (eIF2α), which, on the one hand, attenuates translation initiation to reduce the ER protein-folding load, and on the other hand, stimulates translation of specific mRNA including ATF4. ATF4, in turn, induces transcription of genes encoding ER chaperones, such as BiP and GRP94, UPR-associated transcription factors, such as XBP1, ATF6, and CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP). Among these, CHOP is an important mediator of ER stress-induced apoptosis and oxidative stress and regulator of ER mitochondrial communications (Brewer, 2014).

When there is an accumulation of unfolded or mis-folded proteins in the ER, ATF6 is released from BiP for trafficking to the Golgi apparatus where it is cleaved to yield a cytosolic fragment known as ATF6 p50, which migrates to the nucleus to activate gene expression. This process is termed regulated intramembrane proteolysis (RIP). In the nucleus, homodimeric ATF6 bind to ER stress response element motifs in promoter regions to transactivate ER chaperone genes including BiP. ATF6 can also form heterodimers with XBP-1 to induce the expression of the ERAD components under stress conditions. ATF6 was also proposed to induce ER quality control genes by recruiting the CREB-regulated transcription coactivator 2 (CRTC2) to ER stress-inducible promoters. Hence, ATF6 is essential for optimal protein folding, secretion, and degradation in response to ER stress (Cao and Kaufman, 2012).

As is described above, the three branches of UPR are cross activated by each other. Their activation all contributes to increased expression of ER molecular chaperones, forming a
feedback loop to restore the homeostasis. From the point of view of cybernetics, this mode of transactivation and feedback signaling ensure fine-tuned regulation of the related gene expression to optimize the outcome of the cellular adaptation to environmental changes (Brewer, 2014).

1.2.4 Mitochondrial stress response and mitochondrial UPR (UPR\textsuperscript{mt})

In eukaryotes, mitochondria are the powerhouse of the cells, generating energy via OXPHOS. This energy production process is also concomitant with the generation of the ROS which, under physiological condition and at a low level, play important roles in cell signaling and homeostasis (Yun and Finkel, 2014). This system is so complicated and delicately regulated that constant perturbation occurs due to mismatch of the substrates or changes in the intracellular signaling for different cell behaviors including proliferation, differentiation, stress response to internal or external stimuli, etc. Hence, higher ROS production than normal level and damage of structural and functional molecules in the mitochondria are pervasive and persistent and the molecular chaperones are by no means dispensable and play pivotal roles in the restoration and maintenance of the correct structure of the functional biomolecules in the mitochondria. Moreover, precise maintenance of the mitochondrial proteome is challenged by the partitioning of the protein encoding genes between the mitochondrial and nuclear genomes. Not only that the gene expression in the mitochondria and the nucleus should be concisely coordinated, but also that the nuclear transcribed and cytosolic ribosome translated mitochondrial biomolecules should be efficiently trafficking to the mitochondria to fulfill their...
proper function. This depends on sophisticated mechanisms of mitochondrial protein sorting via mitochondrial targeting sequences (MTS) and the coordinating endeavor of molecular chaperones in the nucleus, cytosol, and mitochondria (D'Amico et al., 2017). Furthermore, optional mechanisms are mandatory when the structural damage of the target proteins is too severe to be repaired. Mitochondrial quality control assisted by mitochondrial molecular chaperones is in charge of such clearance of the damaged proteins (Baker and Haynes, 2011).

It is still not fully understood the trigger and regulation of the mammalian mitochondrial UPR. Owing to the research in C. elegans, in which ATFS-1 is found to be the pivotal regulator of the UPR\textsuperscript{mt}, it has been suggested that ATF5 works in a similar way to control the UPR\textsuperscript{mt} in mammals. In the absence of the mitochondrial stress, ATF5 localizes to mitochondria by MTS and is supposed to be degraded subsequently while under stress conditions, when such a mechanism of ATF5 clearance is impaired, ATF5 localizes to the nucleus to initiate gene transcription to restore the mitochondrial homeostasis (Qureshi et al., 2017). Besides, it has been demonstrated that CHOP is transcriptionally induced during the UPR\textsuperscript{mt} via c-Jun activation to play a role in the mitochondrial quality control mechanism (Horibe and Hoogenraad, 2007). Perturbation of the mitochondrial matrix and intermembrane space (IMS) protein folding environment activates the deacetylase SirT3 to promote mitochondrial recovery through the activation of anti-oxidant machinery and the stimulation of mitophagy which is presumably mediated by the FOXOA3 (Papa and Germain, 2014). Akt activates phosphorylation of the estrogen receptor α is also reported to be protective upon accumulation of unfolded or mis-folded proteins in the IMS (Papa and Germain, 2011). However, further studies are needed to
To establish a better working model to connect all the dots of the above pieces of information together.

The mitochondrial molecular chaperones involved in the UPR\textsuperscript{mt} include mortalin (mtHSP70), HSP10/60, HSP40, TRAP1, and GRP170. As the endosymbionts of prokaryotes origin, HSP10/60 protein folding machinery is conserved to take charge of the proper folding of the proteins in the mitochondrial matrix. Similar with the BiP in the ER, mortalin is the major mitochondrial chaperone elaborating translocation of proteins in and out of the mitochondrial. Also, it works in conjunction with the HSP40 and together with the HSP10/60 protein folding complex, guarantees the timely and precise protein trafficking and communication among nucleus, cytoplasm and mitochondria to ensure the proper function of the organelle (Kaul et al., 2007). TRAP1 is the mitochondrial homologue of HSP90 involved in the maintenance of mitochondrial integrity and protecting cells against oxidative stress and apoptosis. It may also localize at the interface of the ER and mitochondria contact and interact with the proteasome regulatory particle thus involves co-translational quality control of the target proteins (Amoroso et al., 2012; Montesano Gesualdi et al., 2007). Mitochondrial GRP170 is upregulated by CHOP and it is potent chaperone to stabilize and prevent aggregation of damaged proteins due to severe cellular stress (Arrington and Schnellmann, 2008).

It is interesting to notice that, as the important protective and pro-survival transcription factors in UPR\textsuperscript{mt}, CHOP and ATF5 are also activated as pro-apoptotic in the ER stress response. Further research is warranted to answer below questions: 1) Are there different mechanisms of protein structure modification that renders the different function of these transcription factors
in the ER or in the mitochondria? 2) Does this implicate the relationship between the ER UPR and mitochondrial UPR? And how?

It is also intriguing that mitochondrial damage can induce UPR\textsuperscript{mt} in distal tissues by cell non-autonomous signaling through “mitokines” (Durieux et al., 2011). It has been reported that neurotransmitter serotonin and secretory neuropeptide FLP-2 can relay stress signals and stimulate neuronal stress responses in the distal tissues (Berendzen et al., 2016; Shao et al., 2016). This sheds light on a promising novel area of research to elucidate the mechanisms of inter-organ crosstalk in different pathophysiological conditions.

From the point of view of evolution, mitochondria are endosymbionts of prokaryotes’ origin inside the eukaryotic cells. A single eukaryote may contain several thousand mitochondria. The biological interaction between nucleus and mitochondria can thus be taken as communications between thousands of small functional individuals (the mitochondria with a small genome, the function of energy production, and the ability to initiate the persecution of mitophagy) and a single large command center (the nucleus which perceives and integrates the signals from the rest part of the cells and responds by giving orders to synthesize functional and structure molecules accordingly). There exists some uncertainty of the behavior of each single mitochondrion considering the diverging nuclear-mitochondrial communication and the individualized mitochondrial import efficiency, such as seen in PTEN-induced putative kinase 1 (PINK1)-Parkin mediated mitophagy, among mitochondria even within the same cell. Nevertheless, generally applied mechanisms such as the ATF5 induced UPR\textsuperscript{mt} activation are
capable of posing overall impact on whole mitochondria inside the cell. All these contribute to the complexity of the responses and the outcomes of the mitochondria under stress conditions.

1.2.5 Integrated stress response (ISR) determines cell function and cell fate and its implication in the pathophysiology of critical illness

With better understanding of the cytosolic heat shock responses, UPR\textsuperscript{ER} and UPR\textsuperscript{mt}, there has been an increasing appreciation of the cooperation among these different stress response pathways which is termed ISR. This includes cooperation between heat shock response and ER stress/UPR\textsuperscript{ER} (Duennwald, 2015; Liu and Chang, 2008), anterograde and retrograde communication between mitochondria and nucleus (Quiros et al., 2016), interaction between cytosolic heat shock response and UPR\textsuperscript{mt} (Kim et al., 2016a), as well as bidirectional regulation between UPR\textsuperscript{mt} and UPR\textsuperscript{ER} (Li et al., 2006; Takemoto et al., 2011). In the past decade, accumulating evidence has been demonstrating that ISR contributes to various pathophysiological changes in critical illness.

1.2.5.1 ISR in hypoxia and ischemia and reperfusion injury

In hypoxia and ischemia and reperfusion injury, lack of oxygen supply initiates the cellular derangement and impairment of oxidative phosphorylation and significant increase of ROS (Bargiela et al., 2018). This is persecuted and sensed by the mitochondria which crosstalk with the nucleus and the cytosol, activating transcriptional, translational, and post-translational
programs aiming at the restoration of proper mitochondrial function. Such an integrated response is of four-fold. Firstly, decreased cellular oxygen supply results in the impairment of energy production, loss of mitochondrial membrane potential and/or integrity, loss of mitochondrial proteostasis, metabolic dysfunction, and impaired mitochondrial translation, thus activates UPR\textsuperscript{mt} which facilitates the proper translating, folding, and degrading of the mitochondrial proteins within these organelles in response to stress (Jovaisaite and Auwerx, 2015). Secondly, mitochondria are in the process of continuous fission and fusion, which is termed mitochondrial dynamics, to accommodate the cellular metabolic needs and segregate damaged parts from the healthy ones (Wai and Langer, 2016). In conjunction with the quality control mechanisms of mitophagy, homeostasis is restored and preserved by clearance of injured or impaired organelles (Pickles et al., 2018). Thirdly, cytosolic proteostasis networks, including the mitochondria to cytosol stress response (MCSR), mitochondrial precursor over-accumulation stress (mPOS), and the UPR activated by mis-targeting of proteins (UPR\textsuperscript{am}) were recently found to mediate a complex adaptive response to restore cellular protein homeostasis and consequently restore the mitochondrial function, and protect cells from the activation of death signals (Quiros et al., 2016). In the meantime, increased translation of the stress response activated genes also stimulates ER stress and UPR\textsuperscript{ER} especially via the eIF2\textgreek{a} phosphorylation and activation (Baker et al., 2012). Last but not least, it has been observed that, at least in certain types of the cells such as neurons, mitochondrial stress response might signal to distal tissues and organs via mitokines such as serotonin or FLP-2 thus contribute to inter-organ crosstalk of stress responses or even pose impact on changes in epigenetic profile of certain cell types (Ham and Raju, 2017).
1.2.5.2 ISR upon infection and inflammation

Conceptually, I would consider two distinct cellular stress responses upon infection and inflammation: 1) direct cellular stress response upon the insults of pathogens and toxins, 2) indirect or signal transduced stress response upon infection and inflammation.

The direct cellular stress responses upon pathogens and toxins are seen in structural and parenchymal cells attacked by the microbes and/or affected by internal or external toxins. These cells include the epithelia lining as the barrier of the body to the outer environment and endothelia that compose the vasculature, most of the parenchymal cells of visceral organs, muscles, neurons and stromal cells. The initial stress responses in these structural and parenchymal cells upon infection and inflammation are mechanistically similar with the above mentioned cellular stress responses towards hypoxia and ischemia and reperfusion injury. Pathogens and toxins are direct insults to cell structure and function. Cellular stress responses can be activated from any part of the cells depending on the ways the insulting signals are delivered to the cells that are strong enough to trigger the responses. For instance, for epithelial cells of respiratory tract or gastrointestinal tract, the decrease of the innate immunity and the mucosal barrier or the increase of the invasiveness of the pathogens may lead to increased permeability or even destruction of the cell membrane (Naglik et al., 2017; Nowarski et al., 2015); for hepatocytes which are metabolically highly active, the derangement of oxygen or metabolic substrate supply may be first sensed by the mitochondria (Malhi and Gores, 2008). Regardless of the difference in the initiation of the stress response, the cells always respond to the perturbation of the homeostasis in an integrated manner. Specifically, Kim et al. reported
that under the condition of derangement of lipid metabolism with increased synthesis of fatty acids or ectopic fat accumulation, MCSR is activated which involves upregulation of both the UPR\textsuperscript{mt} related genes including dve-1, clpp-1, ubl-5, haf-1 and atfs-1 and HSR related genes like hsf-1 and protects against the proteotoxicity in \textit{C. elegans} and human cells (Kim et al., 2016a).

The major prosecutor of indirect or signal transduced stress response upon infection and inflammation is UPR\textsuperscript{ER}. This is most often seen in immune cells when they detect microbes or damage to tissue integrity to initiate immune responses. Similar cellular stress response can also be seen in some parenchymal cells such as hepatocytes which actively participate in neuronal, hormonal, or intercellular signaling (Szabo et al., 2007). Microbe detection occurs through pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), RNA cytosolic helicases of the RIG-1-like receptors family (RLRs), and NOD-like receptors (NLRs) (Cao, 2016), which sense both conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs), such as Lipopolysaccharides (LPS) or different types of nucleic acids (e.g. ssRNA or CpG DNA) (Akira et al., 2006), and immune-stimulatory products that are derived from damaged tissue or necrotic cells, termed damage-associated molecular patterns (DAMPs) (Qian et al., 2014) which includes high mobility group box protein 1 (HMGB1), IL-33, S100, histones, HSPs, nucleic acids, ATP, and uric acid (Relja et al., 2018). Through the transforming growth factor beta-activated kinase 1 (TAK1), TANK-binding kinase 1 (TBK1), and several IκB kinases (IKK), PRR activation stimulates the expression of interferon-regulatory factor (IRF) and NF-κB transcription factors which, in turn, activate the expression of type-I IFN or pro-inflammatory cytokines, like TNF or interleukin-6 (IL-6) (Li et al., 2011). Metabolic dysregulation, damages to
cell integrity or invading bacteria may also trigger inflammasome assembly and activation in which caspase-1 is activated and, subsequently, potent pro-inflammatory mediators including interleukin 1, 11 and 18 (IL-1, IL-11, and IL-18) are processed and secreted (Lamkanfi and Dixit, 2014). In these processes, due to the increased synthesis and folding of secreted and transmembrane proteins, there is an accumulation of nascent, unfolded proteins in the ER. Meanwhile, different stressors, such as ROS or bacterial toxins, can lead to the accumulation of mis-folded proteins. UPRER is thus triggered which favors ER proteostasis and promotes cell survival (Moore and Hollien, 2012).

In recent years, it has been demonstrated that there is a synergistic interaction between the UPR and inflammatory cytokine production in the immune cells, in which three different branches of the UPR crosstalk with specialized microbe sensing pathways to turn on or amplify inflammatory cytokines production (Reverendo et al., 2018).

IRE1 signaling pathway was shown to be essential for immunity, since efficient immune responses against pathogens cannot be induced in C. elegans and mice lacking XBP1 (Martinon and Glimcher, 2011). Accumulation of several metabolites, such as fatty acids or cholesterol, triggers IRE1 signaling and amplifies pro-inflammatory responses, further illustrating the synergistic interaction between PAMP/DAMP sensing pathways and this specific UPR signaling branch (Fu et al., 2012). XBP1 activation is sufficient in macrophages and stromal cells to drive pro-inflammatory TNF and IL-6 transcription (Martinon et al., 2010). IRE1 can also promote phosphorylation and activation of the JNK and of NF-κB, through direct binding to the TRAF2 in the cytoplasm (Urano et al., 2000). Moreover, IRE/XBP1 signaling pathway has important
regulatory functions for the differentiation of the both B cells and T cells (Brunsing et al., 2008). It has also been shown that IRE-XBP1 signaling may activate cellular non-autonomous transcriptional regulation in hepatocytes which results in enhanced insulin sensitivity and glucose regulation (Williams et al., 2014). Similar regulatory effect of XBP1 on inflammatory response is also seen in endothelial cells (Gargalovic et al., 2006).

PERK-eIF2α branch, regarded as important signaling pathway for ISR, proves to be one of the crucial links between ER and inflammation in several studies (Guthrie et al., 2016; Halliday et al., 2017). PERK activation also promotes NF-κB translocation and pro-inflammatory cytokines transcription in stressed cells, while the phosphorylation of ISR and eIF2α leads to a profound reduction of protein synthesis which potentiates pro-inflammatory signaling by impacting several short-lived signaling inhibitors, such as IκBα, that depend on neosynthesis to exert their activity. Such a timely re-estabishment of IκBα is essential to the negative feedback signaling loop that ends NF-κB activation and prevents hyper-inflammation after microbe sensing (Deng et al., 2004).

The role of ATF6 signaling pathway on inflammation is least studied so far in the three branches of UPR. Since ATF6 and XBP1 can form heterodimers and regulate the gene expression in stressed cells, it may contribute to the balance between protein folding and degradation thus impact the immunological function of XBP1. It has been shown that the ATF6 branch synergizes with TLR stimulation to potentiate a pro-inflammatory phenotype with enhancement of NF-κB signaling in liver macrophages (Rao et al., 2014).
In the above mentioned cellular stress response upon infection and inflammation, evolutionary pressure is in favor of the integration of the inflammatory response with the metabolic regulation since a strong immune response is highly energy-dependent. Indeed, evolutionary conservation of the immune and metabolic pathway crosstalk is clearly demonstrated in the *Drosophila* fat body, which is the functional equivalent to the liver and adipose tissue in mammals and serves to both sense and store nutrients and defend against pathogens and immune mediators, such as cytokines, are envisioned as metabolic hormones (Hotamisligil, 2017). Such multi-functional mediators or signaling molecules include but are not limited to TNFα, IL-1, and IL-6 (Varela et al., 2018).

### 1.2.5.3 ISR in cell death and tissue and organ damage

When the UPR\textsuperscript{ER}, UPR\textsuperscript{mt} and cytosolic HSR cannot accommodate the stressful perturbation of the cells, the cell death signaling is activated. Depending on the mode of ISR and, in turn, the different ways of how such cell death signaling pathways are activated, the outcome of the cell death can be either programmed cell death (including apoptosis, necroptosis, and pyroptosis) or necrosis which has different implications on tissue and organ damage (Galluzzi et al., 2007).

Multiple studies indicate the interaction between ER stress and UPR\textsuperscript{ER} and cell death signaling. The protein kinase domain of IRE1 activates JNK via interaction with TRAF2 and ASK1 which contributes to the induction of apoptosis. Activation of PERK leads to phosphorylation of eIF2α and causes general inhibition of translation of multiple genes on the one hand and
selective activation of the transcription factor ATF4 and thereby enhanced pro-apoptotic CHOP expression (Kim et al., 2006).

Considering the endosymbiotic nature of the mitochondria in the mammalian cells (Dyall et al., 2004), it is well accepted that mitochondria play a central role in mammalian cell death, not only because their disruption results in an energy crisis, but also because they harbor factors that actively promote some types of cell death, such as mitochondrial permeability transition (MPT)-driven necrosis (Cheng et al., 2008). However, on the one hand, since UPR\textsuperscript{mt} involves activation of the CHOP transcription and JNK signaling pathway which play a role in mitochondrial quality control mechanisms and it has been observed that the increased mitochondrial fission is correlated with the cell death (Frank et al., 2001), it is reasonable to postulate that UPR\textsuperscript{mt} may contribute to the cell death signaling especially when under aggravated mitochondrial stress conditions. On the other hand, impaired UPR\textsuperscript{mt} may result in the aforementioned mitochondrial precursor over-accumulation stress (mPOS), leading to the aberrant accumulation of mitochondrial precursors in the cytosol which may trigger cytosolic proteostatic stress and cell death (Wang and Chen, 2015).

As the result of the aggravated stress response, the cell death contributing to the overall outcome of the tissue and organ damage is partly determined by the interaction of the UPR\textsuperscript{mt}, UPR\textsuperscript{ER} and cytosolic HSR, with the apoptosis on the one end in which the pro-apoptotic signaling is well coordinated among the cellular compartments and the necrosis on the other end in which the insult and the derangement of the cellular homeostasis is so intense that the cells die without any effective compensatory reactions. In between these two scenarios,
necroptosis is the cell death without the caspase activation but induced by receptor interacting protein kinase 1, 3 (RIPK1, RIPK3) and their substrate mixed lineage kinase like (MLKL) (Pasparakis and Vandenabeele, 2015) whereas pyroptosis is defined as highly inflammatory and pertaining to cell death as the result of the inflammasome activation (Galluzzi et al., 2018).

1.2.6 Summary

As one of the fundamental instincts of the living organisms, cellular stress response is highly conserved among the species. A group of conserved macromolecules, namely molecular chaperones, prosecute the response. To ensure the prompt feedback to the changing of the environment, the mode of such a response is not through the activation of the genes encoding these molecular chaperones but rather, quite a few important molecular chaperones are constitutively expressed and bind to and stabilize the key signaling modulators of the cellular stress response related genes. This way, when there is a perturbation of the homeostasis, these molecular chaperones not only work as first responders to rescue the cellular homeostasis, but also free up their gene expression modulators to activate subsequent cellular stress responses.

In eukaryotes, cellular stress response is compartmentalized into cytosolic HSR, ER stress response and UPR\textsuperscript{ER}, and mitochondrial stress response and UPR\textsuperscript{mt}, responding differentially to various stimuli, cross-talking and integrating the signals to pose overall impact on the cell function and cell fate.
In multi-cellular organisms, specifically in mammals, upon perturbation of homeostasis, cellular stress response is activated to rescue the cell structure and function and, when failed, is quickly shifted to initiate programmed cell death to minimize the impact of the insult to the rest part and the whole organism.

Pertaining to thermal injury, profound and pervasive cellular stress responses in multiple organs and systems are the most pivotal pathology to be investigated. Such cellular stress responses are triggered by multiple internal and external stimuli (including hypovolemic shock, ischemia and reperfusion injury, infection, etc.), interact with inflammation in the wounds, the lung, the liver, and the gastrointestinal tract, thus impose great impact on the systemic pro-inflammatory responses, metabolic changes, and the final outcome of the injury. Experimental studies of cellular stress responses in the context of perturbation of homeostasis in major burn injury are thus of important clinical significance.

1.3 Hepatic immunometabolic disorder, liver damage and regeneration after severe trauma injury

1.3.1 Immunometabolism and hepatic inflammasome activation under stress conditions

With the advancement of the diabetes research and the increasing understanding of the pathology of chronic inflammation and its contribution to metabolic dysfunction, the concept of immunometabolism is well established for the interplay between immunological and metabolic
processes (Mathis and Shoelson, 2011). The majority of the early studies of immuno-
metabolism focus on metabolic regulation of the immune cell functions including 1) the
elucidating of the association between the different immune cell functions and their distinct
metabolic configurations, such as the ATP generation via OXPHOS in resting immune cells
versus aerobic glycolysis in stimulated macrophages and antigen activated T cells, and 2) the
analysis of the metabolic reprogramming of immune cells which leads to changes in their
functional properties, such as the shift from M1 to M2 upon the inhibition of glycolysis (Norata
et al., 2015). It has been well accepted that the cells requiring rapid division cycles or activation
as seen in oncogenically transformed malignant cells and embryonic stem cells import and burn
massive amounts of glucose to generate biomass (Warburg-type metabolism), whereas cells
destined for quiescent or surveillance roles generally use fatty acid oxidation and the Krebs
cycle to generate energy (Murray et al., 2015).

Specifically, T effector cells emerge from quiescence following activation by antigen
through the T cell receptor and co-stimulation through an mTOR-dependent process that
involves an increase in aerobic glycolysis (Warburg-type metabolism). Activated T effector cells
undergo multiple cellular divisions and convert glucose and glutamine into biomass, require
substantial supplies of amino acids imported from the local environment, and are dependent
on TORC1, PI3K, and Akt. Glycolysis also allows the efficient translation of mRNAs encoding
effector cytokines, such as IFN-γ. To the other end, memory T cells maintain healthy
mitochondria by synthesizing and then oxidizing fatty acid to support OXPHOS thus enable the
cells to persist for long periods as quiescent cells. Regulatory T cells are dependent on FoxP3,
allowing highly specific genetic tests of regulatory T cells metabolic requirements. Mice lacking mTOR in all T cells have a phenotype similar to regulatory T cells, arguing that mTOR signaling counters the regulatory T cell phenotype. Similarly, Akt activation blocks regulatory T cells. However, ablation of Raptor (a component of TORC1) using FoxP3-Cre causes defects in regulatory T cell number and function. It thus appears that regulatory T cells development and function are context-dependent on specific metabolic cues: regulatory T cells expansion likely requires TORC1 and glycolysis, though this pathway must be suppressed for them to become fully functional. Moreover, withdrawal of essential amino acids such as arginine is thought to help T effector cells to convert to a more regulatory state (MacIver et al., 2013). For B cells, although much less is known about how metabolism directs the fate of normal B cells (Pearce and Pearce, 2013), it is suggested that upon activation, glycolytic metabolic pathways and glutamine metabolism are stimulated and endoplasmic reticulum is expanded to make and secret antibodies (Le et al., 2012). Also, B cell receptor activation is regulated by ROS (Wheeler and Defranco, 2012) and along with ROS signaling, new studies also suggest that other mitochondrial-derived molecules play important roles in B cell activation and effector function (Weinberg et al., 2015).

Immunometabolic regulation of macrophage has also been intensively investigated. M1 macrophages activated by pathogen products and type I IFNs are glycolytic and anti-microbial and have tissue-destructive potential whereas M2 or alternatively activated macrophages have a different metabolic profile and inflammatory phenotype of increased flux through OXPHOS and higher expression of anti-inflammatory cytokines such as IL-10, but decreased production
of NO and TNF-α. Since most M1 macrophages in inflammatory sites originate from bone marrow inflammatory monocytes, they are replaceable with no need of self-renewal. M1 macrophage features increased production of NO thus requires the import of arginine and oxygen. iNOS mediated nitric oxide synthase reaction produces citrulline and NO and citrulline, in turn, is exported and then re-imported as needed to re-generate arginine and sustain NO production forming the cycle as part of the anaplerotic TCA cycle of M1 cells, which can also lead to poisoning of mitochondrial respiratory activity. In addition to NO, major products of M1 macrophages are cytokines, chemokines, metalloproteases, and the anti-microbial metabolite itaconate. The TCA cycle is fragmented in M1 macrophages, and this is associated with an accumulation of succinate, which has pro-inflammatory effects by stabilizing HIF1α. M2 macrophages do not make NO and instead use Arg1 induced by a TLR-dependent indirect mechanism to hydrolyze massive amounts of imported arginine. While the products of this reaction are ornithine and urea, the main function of arginine consumption by M2 macrophages is to restrict supply to neighboring arginine auxotroph: M2 macrophages are therefore immuno-regulatory and may suppress T effector cells by blocking their supply of arginine. M2 macrophages use FA primarily derived from acquired triacylglycerols to support OXPHOS, have an intact TCA cycle, and make large amounts of glycosylated proteins thus requiring UDP-GlcNac from glucose. However, like monocyte-derived macrophages, tissue macrophages can be M1 or M2 polarized, depending on the inflammatory micro-environment (Kelly and O’Neill, 2015).
In recent years, there is a growing appreciation of the intercellular crosstalk and its contribution to pathological changes upon perturbation of homeostasis. Pertaining to the liver pathology, since the hepatocytes and Kupffer cells are the two major cell populations, their interactions under stress conditions are supposed to be bi-directional: not only that hepatic metabolic derangement contributes to Kupffer cells metabolic reprogramming thus interferes with the innate immunity as is suggested in the above mentioned immunometabolic regulation; but also that Kupffer cells activation upon stress may pose significant impact on hepatic metabolic dysfunction, thus forming a dynamic loop of immunological and metabolic interplay.

The most important progress of the understanding of the innate immune response under stress conditions is the elucidation of the mechanisms of inflammasome activation and consequent pyroptosis (Latz et al., 2013). The inflammasomes are organized by sensor molecules connecting to caspase 1 via ASC, which is an adaptor protein encoded by PYCARD and consists of two death-fold domains: one pyrin domain and one caspase activation and recruitment domain (CARD). ASC interacts with the upstream inflammasome sensor molecules via the pyrin domain. Upon receiving of activation signals, such interaction between ASC and sensor molecules triggers the assembly of ASC into a large protein speck consisting mainly of multimers of ASC dimers, bringing monomers of pro-caspase 1 into close proximity, which initiates caspase 1 self-cleavage and the formation of the active hetero-tetrameric caspase 1. Active caspase 1 proteolytically activates a number of proteins including pro-IL-1β and pro-IL-18. Pro-IL-1β is transcriptionally induced by the transcription factor NF-κB whereas pro-IL-18 is constitutively expressed. Nevertheless, these potent pro-inflammatory cytokines are controlled
by two checkpoints of transcription as well as maturation and release. One of the most important inflammasome sensor molecules is NLRP3 especially if considering the hepatic immunometabolic interactions since it senses mitochondrial stress including the excessive production of ROS and oxidized mitochondrial DNA (Zhou et al., 2011). Moreover, since NLRP3 basal expression is not sufficient for inflammasome activation in resting cells, NLRP3 inflammasome activation requires a priming step and a second activation step which renders multi-checkpoints regulation of its activation.

In the context of severe thermal injury where profound stress response is followed by prolonged immunological and metabolic derangement, it is necessary to investigate the interaction between the hepatic inflammasome activation and metabolic dysfunction which could implicate the linkage among these pathological processes. Furthermore, the increased infiltration of the macrophages and adrenergic stimulation of lipolysis in the WAT are evident. It is also of mechanistic significance to investigate the inflammasome activation in the adipose tissue upon stress conditions post-burn and its contribution to overall metabolic disorder after burn injury.

1.3.2 Liver regeneration under profound stress condition and severe liver damage

It is well accepted that the liver is a regenerable organ and, in the normal liver, there is a population of 0.0012% to 0.01% mitotic hepatocytes contributing to the renewal of the whole parenchyma in about one year (Christ and Pelz, 2013). The idea of mature hepatocytes
duplication and proliferation for the physiological hepatic parenchymal renewal in adults has recently been mainstreamed (Yanger et al., 2014). However, it is still impossible to conclude such physiological hepatic renewal by mature hepatocytes until rigorous and confirmative experiments further exclude the presence of stem cells in the liver (Miyajima et al., 2014). Indeed, hepatic maturational lineages of cells beginning extra-hepatically in the hepato-pancreatic common duct and intra-hepatically in periportal triads as well as the streaming theory of stepwise maturation and migration of hepatocytes from periportal region to peri-central region are still supported by multiple lines of experimental evidence (Cardinale et al., 2012; Turner et al., 2011).

It is believed by many that after massive liver injury, progeny of the putative liver stem cells appear in the periportal triads of the liver lobules which have been identified as the Canals of Hering, the most proximal parts of the intra-biliary ductular system. In humans, such stem cell proliferation response with the formation of the tubular structures appears after severe liver injury was termed “ductular reaction”, which is equivalent to the appearance and proliferation of oval cells in rodents (Christ and Pelz, 2013). However, this conjecture is also challenged by the well-designed chimeric lineage tracing experiments which demonstrated the reversible ductal metaplasia from mature hepatocytes in response to injury and its contribution to the subsequent liver regeneration (Tarlow et al., 2014b).

Considering all the above controversies in the liver regeneration under physiological condition as well as upon liver injury, a considerable degree of plasticity in the liver is proposed, whereby several cell types can contribute to regeneration (Hindley et al., 2014).
The question followed would be how the cell proliferation and differentiation is stimulated in the context of cellular stress responses, especially in those facultative liver stem cells which are usually quiescent under physiological conditions. It has been suggested that the activation of the ER stress and the UPR\textsuperscript{ER} is not only a mechanism for the eliminating of stem cells that encounter insults during the development but also beneficial for directing stem cells into proliferation and differentiation in neuronal stem cells (Kawada et al., 2014), bone marrow mesenchymal stem cells (Yu et al., 2013), and myoblast (Alter and Bengal, 2011). Moreover, recent findings have revealed the pivotal role of mitochondria in stem cell fate decision and function through the mechanisms of metabolic modulation and retrograde signaling by metabolites from multiple metabolic pathways including TCA cycle, OXPHOS and ROS, fatty acid \(\beta\)-oxidation, and one-carbon cycle (Anso et al., 2017; Buck et al., 2016; Jin et al., 2018; Khacho et al., 2016; Zhang et al., 2016). However, since the roles of ER stress, UPR, and mitochondrial modulation on the stem cell function and fate decision are cell-type dependent, further study is needed to investigate how these pathways regulate the liver stem cell proliferation and differentiation (Yang et al., 2016; Zhang et al., 2018).

To clarify and clearly elucidate the mechanisms of liver regeneration, we need to overcome the obstacle of labeling and tracing the different hepatic cell types with high efficiency and fidelity (Miyajima et al., 2014). To this end, and owing to the increased understanding of the hepatic and gastroenterological embryology, Sry (sex determining region Y)-box 9 (Sox9), a member of the Sry-related high-mobility group box transcription factors, was recognized as fundamental in maintaining cells in an undifferentiated state and capable of
marking the precursor cell population during physiological cell replacement and/or during the regenerative process after injury (Furuyama et al., 2011). By combining the floxed reporter allele of fluorescent protein whose expression is activated by Cre-mediated recombination (such as ROSA26r, in which a loxP–STOP–loxP–EYFP gene cassette is knocked in to the ubiquitously expressed ROSA26 locus), Sox9-Cre can be fused with the ligand-binding domain of the estrogen receptor (CreER), rendering its EYFP expression tamoxifen inducible. Because recombination occurs within the genome DNA, this mark is inherited by all descendants of the labeled cells regardless of its subsequent cell fates (Kopp et al., 2011). Multiple experimental studies demonstrated the efficacy of this system in tracing the hepatic progenitor cells under various conditions (Font-Burgada et al., 2015; Kawaguchi, 2013; Tarlow et al., 2014a).

Considering that the Sox9-Cre/ERT2: EYFP system marks all descendants of the labeled cells regardless of its subsequent cell fates, another marker to distinguish between progenitor cells and their progeny is needed. It has been well demonstrated that EpCAM is a suitable candidate since it is highly expressed on tissue progenitors, and embryonic and adult stem cells, but gradually lose the expression along with maturation into hepatocytes (Dolle et al., 2015).

With the awareness of the profound perturbation of homeostasis after major thermal injury, the significantly increased metabolic challenges, and multiple comorbid factors including wound and systemic inflammation/infection, it is reasonable to postulate the stimulation of liver regeneration after major burn injury. If so, it is of clinical significance to better understand how such liver regeneration is initiated, how long does it last, and how does it resolve with the recovery of the burn injury. Furthermore, we would speculate that liver regenerated under
stress conditions after major burn injury may behave differently as compared with those mature hepatocytes and hepatocytes regenerated under physiological conditions. It is interesting to see if facultative hepatic stem cells proliferate and differentiate under stress conditions are configured metabolically stressful, and if so, whether such metabolic stress could be passed on to their progeny, contributing to the persistent metabolic disorder post-burn. The research in this discipline might pave the way to the discovery of novel therapeutic intervention.

1.4 Research problem, rationale and working hypotheses

1.4.1 Research problem and rationale

Burn injury represents one of the most severe forms of trauma (Brigham and McLoughlin, 1996) and metabolic dysfunction is common after severe burn injury, persisting for several years and contributing to significant morbidity and mortality (Herndon and Tompkins, 2004; Jeschke et al., 2008a). As the functional hub of immunologic response, metabolic response, inflammatory response and acute phase response, liver is fundamental in the pathology of post-traumatic metabolic dysfunction. Significant hepatomegaly is universally evident in extensively burned patients. This implicates both hepatic lipid infiltration, which may augment hepatic ER stress and impair liver function, and the abnormal hepatocyte proliferation and regeneration correlated with metabolic perturbation (Jeschke et al., 2004; Jeschke et al., 2001). Experimental studies are warranted to understand the underlying mechanisms of liver
dysfunction and damage as well as its contribution to prolonged inflammation and hypermetabolism in severely burned patients.

On the one hand, hepatic stress response and fat infiltration might contribute to metabolic dysfunction post-burn. Pervasive and profound stress responses are presented after major burn injury due to pathophysiological consequences including hypovolemic shock, tissue damage and loss of skin barrier thus wound infection and sepsis, significant higher metabolic demand and substrate mobilization for wound repair. It is reasonable to speculate that hepatic ER stress contributes to liver dysfunction and liver damage. Furthermore, recent evidence indicates that stress signals transferred into adipose tissue stimulate lipolysis (Bogdanovic et al., 2015). Consequently, we postulated that elevated level of circulating free fatty acid (FFA) and increased efflux of lipid to the liver contribute to hepatic fat infiltration and augment hepatic stress response and organ damage.

On the other hand, hepatocytes regenerated under stress conditions might be configured to pro-inflammation and/or hypermetabolism. It is known that liver is a regenerable organ and it takes approximately one year for the renewal of the whole hepatic parenchyma (Christ and Pelz, 2013). This suggests that liver contains stem/progenitor cells in an environment that enables regeneration (Miyajima et al., 2014). It has also been shown that the cell susceptibility to stress is divergent among stem cells, progenitor cells and differentiated cells (van Galen et al., 2014). Burns, like other stressors, induces accumulation of unfolded or misfolded proteins in the ER and consequently activates UPR. This enables cells to either resolve the stress or move toward apoptosis. It is important to know if the susceptibility of
stem/progenitor cells in the liver is different from mature hepatocytes. Burn patients stay in hypermetabolic state for several years after thermal injury. This suggests that: 1) hepatic stem/progenitor cells might be more susceptible to ER stress; 2) there might be increased proliferation of this group of cell post-burn; 3) stress signals might lead to metabolic derangement this group of cells; and 4) the hepatic stem/progenitor cells and their progeny may retain the stress-induced metabolic derangement profile and thus contribute to prolonged pro-inflammatory responses and hypermetabolism (Heard and Martienssen, 2014; Tsankov et al., 2015).

1.4.2 Hypotheses and specific aims of the study

In the current PhD program, I hypothesize that 1) there are pervasive cellular stress responses after severe burn injury; 2) in the WAT, such cellular stress response stimulates lipolysis, contributing to hepatic fat infiltration; 3) in the liver, hepatic cellular stress response and hepatic fat infiltration stimulate inflammasome activation, induce hepatic metabolic dysfunction and liver damage; 4) hepatic cellular stress response and liver damage trigger facultative liver stem cells proliferation and differentiation which give rise to metabolically dysregulated hepatocytes, contributing to persistent hypermetabolism.

I am conducting experimental studies with below specific aims (Figure 1.1):

Specific Aim 1: Determine if increased lipolysis in WAT is the pre-hepatic mediator of metabolic stress after thermal injury.
To test if severe burn injury leads to ER stress, inflammasome activation, and apoptosis in WAT, thus stimulates lipolysis, contributing to hepatic fat infiltration. (Chapter 2)

Specific Aim 2: Determine the intra-hepatic pathological changes after thermal injury.

1) To test if severe burn injury leads to hepatic stress response including ER stress, mitochondrial dysfunction and inflammasome activation, contributing to liver damage and dysfunction. (Chapter 3)

2) To test if chronic hepatic stress as a result of obesity in tandem with the acute perturbation of homeostasis post-trauma contributes to worse clinical outcomes due to augmented hepatic fat infiltration, cell damage and impaired liver function. (Chapter 4)

Specific Aim 3: Determine if and how stress induced liver regeneration contributes to persistent metabolic dysfunction after thermal injury.

To test if there is increased liver regeneration from facultative liver stem cells after major burn injury and if the liver parenchyma regenerated under stress condition is different from physiological renewal and hepatocytes regenerated under such stressful condition carry on dysfunctional metabolism and pro-inflammatory signaling, contributing to prolonged inflammatory responses and hypermetabolism. (Chapter 5)
Figure 1.1 Specific aims of the study.
Chapter 2  Lipolysis in WAT and Its Contribution to Hepatic Fat Infiltration

This chapter is adapted from the below published original research article:


2.1 Introduction

To better understand the liver-WAT crosstalk after major burn injury and the mechanisms of stress-induced lipolysis in WAT as a pre-hepatic factor, contributing to the hepatic fat infiltration, we conducted animal experiment using a 2-hit rat model of 60% total body surface area (TBSA) scald burn plus sub-lethal LPS intraperitoneal injection (10mg/kg body weight) to mimic major burn injury plus septic response seen in severely burned patients.

Major burn injury represents one of the most severe forms of trauma and features pervasive stress responses at cellular, tissue and systemic level (Jeschke and Boehning, 2012; Jeschke et al., 2012). Such stress responses are usually followed by wound infection, systemic inflammatory responses and sepsis that augment the severity of metabolic dysfunction and immunological impairment in the patient (Jeschke et al., 2014b). Persisting catabolic hypermetabolism, including insulin resistance (IR), hyperglycemia and lipolysis, is the common
clinical manifestation in severely burned patients, and these responses are augmented when sepsis is present (Jeschke et al., 2014a). This can lead to impaired immune function, poor wound healing, and can increase the incidence of multiple organ dysfunction/failure and even death (Herndon and Tompkins, 2004; Jeschke et al., 2008a). Using a 2-hit rat model of burn injury plus lipopolysaccharide (LPS) injection, we observed increased fat infiltration in liver tissue. This is consistent with the clinical findings that significant hepatic fat infiltration and hepatomegaly are associated with increased incidence of sepsis and mortality in severely burned patients (Barrow et al., 2005; Jeschke, 2009). However, the mechanisms underlying the abnormal deposition of lipid in the liver are unclear. Due to the increased appreciation of metabolic cross-talk between liver and WAT in the context of IR and hypermetabolism (Glass and Olefsky, 2012), we hypothesized that increased lipolysis in WAT after severe burn injury and sepsis contributed to hyperlipidemia and hepatic lipid infiltration which led to detrimental outcomes.

The aim of the current study was to determine the underlying mechanisms of lipolysis post-burn using a two-hit model of burn plus LPS. We also examined whether hepatic steatosis is linked to pathological changes in WAT.
2.2 Materials and Methods

2.2.1 Animal model

Animal experiments were approved by the Animal Care and Use Committee of Sunnybrook Research Institute in Toronto, ON. The National Institutes of Health Guidelines for the Care and Use of Experimental Animals were met.

Male Sprague Dawley rats (n=8 per group), 275 to 300 grams, were purchased from Charles Rivers Laboratory International Inc. (MA, USA) and were allowed to acclimate for 1 week before experiments. Rats were housed in the Sunnybrook Research Institute animal care facility and received a high protein diet (Ensure, #22017C8, Abbott Laboratories, IL, USA) and water *ad libitum* from arrival until sacrifice. Ensure was given from 7 days before the study to adjust the animals to the liquid diet.

The animals were randomized into 4 groups: sham, burn only, LPS only and burn plus LPS. A well-established method was used to induce a full-thickness scald burn of 60% TBSA (Herndon et al., 1978; Jeschke et al., 2011b). Briefly, the animals were anesthetized with intraperitoneal injection of ketamine (40mg/kg) and xylazine (5mg/kg), shaved on both the dorsum and the abdomen, and then placed in a mold that exposed a defined area of skin. The exposed skin was lowered into water of 98°C for 10 seconds on the back and 1.5 seconds on the abdomen to induce full-thickness scald burn. The animals were monitored and taken care of accordingly post-burn. Since the confirmed full-thickness burn was applied, analgesia was usually not indicated after burn injury. Lactated Ringer’s solution (30ml/kg) was administered
intraperitoneally immediately after the burn for resuscitation. Sham animals were anesthetized and shaved but not burned. The second hit of an intraperitoneal injection of LPS from *Pseudomonas Aeruginosa* (10mg/kg, Sigma, St Louis, Mo) was applied 72 hours post-burn. All the animals were sacrificed 24 hours after LPS injection (96 hours post-burn).

### 2.2.2 Cell culture

3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mM L glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO$_2$ at 37 °C. Differentiation of the cells to mature adipocytes was performed as described previously using a differentiation cocktail containing Rosiglitazone (Zebisch et al., 2012). Adipocytes were serum-deprived overnight before the experiment. After the treatment, the cells were briefly washed with phosphate-buffered saline (PBS), and then preserved immediately at -80 °C for future analysis.

### 2.2.3 Plasma and tissue collection

Blood was collected into EDTA-containing tubes (30 μL of 0.5 M EDTA). The tubes were placed on ice for at least 30 min and centrifuged at 4°C for 10 min at 1000 × g. The plasma supernatant was aliquoted and stored at -80°C for later analysis. Livers were collected after brief portal vein perfusion with PBS (10 mL). Liver and WAT were frozen immediately on dry ice and then stored at -80°C for gene expression and Western blot analyses. Tissue samples were fixed in 10% buffered formalin at 4°C overnight, transferred to 70% ethanol and then paraffin
embedded for histology. A second set of tissue samples were embedded in OCT compound (VWR Cat No: 95057-838), frozen on dry ice and stored at -80 °C for Oil-Red-O staining.

2.2.4 Gene expression analysis

Total RNA was isolated from liver following manufacturer’s instructions (RNeasy Mini Kit; Qiagen, Hilden, Germany), quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and reverse transcribed (Applied Biosystems, San Diego, CA, USA). Real-time quantitative PCR was performed on cDNA with the housekeeping gene rRNA 18S. The sequences of primers of ER stress marker gene CCAAT/-enhancer-binding protein homologous protein (Chop): 5’- AGCGCCTGACCAGGGAGGTA and 5’- GCTTGGCACTGCGTGATGGT-3’.

2.2.5 Western blotting

Antibodies against rat total and phosphorylated AMP-activated protein kinase (AMPK and phospho-AMPKα), total and phosphorylated protein kinase A catalyst unit (PKA C and phospho-PKA C), hormonal-sensitive lipase (HSL, total and phosphorylated at Ser563, 565, 660 respectively), phosphorylated p44/42 MAPK, perilipin, CHOP, caspase-3, caspase-9, cleaved caspase-3, cleaved caspase-7, GAPDH and tubulin were purchased from Cell Signaling (Danvers, MA, USA). Anti-NOD-like receptor, pyrin domain containing 3 (NLRP3) and anti-ATF6 antibodies were purchased from EMD Millipore (Billerica, MA, USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific Inc. (Rockford, IL, USA).
Approximately 50 mg of frozen WAT tissue and whole cell lysates (50µg of protein per well) were homogenized in 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% (w/v) NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 2.5 mM sodium pyrophosphate, and 1x Complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN, USA). The homogenate was centrifuged at 12,000 ×g for 30 min at 4°C and the pellet discarded. Protein samples (50µg of protein per well) were separated by 10% SDS-PAGE gel and then transferred to nitrocellulose membrane and blots were probed using the antibodies listed above. Band intensities were detected, normalized and quantified with the Chemidoc and Image Lab 5.0 software (Bio-Rad Laboratories, Hercules, CA). GAPDH and tubulin were used as loading controls depending on molecular weights of the target proteins.

2.2.6 **Immunofluorescent multi-channel staining of WAT**

Antibody staining was performed as described previously (Amini-Nik et al., 2014). Primary antibodies against perilipin, cleaved caspase-3 and CHOP were purchased from Cell Signaling (Danvers, MA, USA). Fluorophore secondary antibodies (Alexa Fluor® 647 Donkey Anti-Mouse and Alexa Fluor® 488 Goat Anti-Rabbit) were purchased from Life Technologies (Carlsbad, CA, USA). Multiple negative controls of blank, first or second antibodies alone were set up in each batch of experiment to deduct the auto-fluorescence of the WAT. Mounting media with DAPI was applied for nuclear staining. The percentage of marker-positive cells was determined by taking representative images and directly counting cell number. Cell enumerations for each experiment are listed in the text or figure legends.
2.2.7 Hematoxylin and eosin (H&E), Oil Red O (ORO), immunohistochemical (IHC) and TUNEL staining of tissue sections

H&E and ORO staining of WAT as well as IHC staining of perilipin in liver were performed as described previously (Arno et al., 2014; Bogdanovic et al., 2015). The size of adipocytes was measured using Image J v1.48 (NIH, USA). TUNEL staining of WAT was performed using an immunofluorescent TUNEL staining kit following the manufacturer’s instructions (Promega, Madison, WI, USA).

2.2.8 Determination of free fatty acid (FFA), glycerol and triglyceride levels in blood

Levels of FFA, glycerol and triglyceride in the blood were determined using FFA, glycerol and triglyceride colorimetric assay kits according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, Michigan, USA).

2.2.9 Statistical analysis

The statistical analysis was performed using Prism version 5.01 (GraphPad Software, San Diego, CA). One way ANOVA was performed with Bonferroni posttest, p < 0.05 was considered statistically significant.
2.3 Results

2.3.1 Burn and LPS induce significant catabolism and hepatic fat infiltration

We observed significant weight loss in the LPS and the burn plus LPS groups compared to the sham controls, but there were no changes in body weight in the animals subjected to burn alone (Figure 2.1, A). IHC staining of perilipin and ORO staining of neutral lipid in liver indicated increased lipid accumulation in the burn and LPS groups; however, we observed a synergistic effect of burn plus LPS on perilipin and neutral lipid in liver (Figure 2.1, B-E). Observation of increased hepatic steatosis corroborates previous findings and validates our 2-hit animal model, implicating catabolism and robust lipid mobilization from WAT after burn plus LPS.
Figure 2.1  Burn and LPS induced catabolism and increased liver fat content.

Weight gain/loss (A) was presented in percentage of pre-experimental weight. Representative images of perilipin IHC in liver were presented in (B). Arrows indicate perilipin staining. Scale bar=50μm. Representative images of ORO staining of liver were presented in (C). Arrowheads indicate positive ORO staining. Scale bar=50μm. Percentage of perilipin positive and ORO positive hepatocytes was presented in (D) and (E). Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test). N=8 animals per group, N=2 for histological observations in each group.
2.3.2 Increased WAT lipolysis in the 2-hit rat model of burn plus LPS

We found lower EWAT mass (data not shown) as well as a smaller median adipocyte cell size in burn, LPS, and burn plus LPS groups compared to sham (Figure 2.2, A). The median adipocyte size in sham animals was 2500µm², whereas 90% of adipocytes in burn, LPS, and burn plus LPS groups were smaller than 2500µm² (Figure 2.2, B). Furthermore, immunofluorescent staining of WAT sections (Figure 2.2, C) and Western blot analysis of WAT (Figure 2.2, D-E) demonstrated that the perilipin coating of adipocytes in burn, LPS and burn plus LPS animals was significantly compared to sham, supporting the notion that WAT lipolysis was exacerbated in the 2-hit rat model (Grisouard et al., 2012).
Figure 2.2  Decreased adipocyte cell size and perilipin content in WAT of rats subjected to burn plus LPS.

Representative images (A) depicted H&E staining of EWAT. Arrows indicate smaller adipocytes in burn, LPS and burn plus LPS as compared to sham; arrowheads indicate cell infiltration in WAT. Scale bar=100μm. Measurement of adipocyte cell size was presented in (B), 1000 cells per group. Values are means ± SEM (shown in brown and red bars). *P<0.05 (one-way ANOVA, Bonferroni multiple comparison test). Immunofluorescent staining of perilipin in EWAT was presented in (C) (magnification ×400). Representative images of Western blot (D) and quantitative densitometric analyses (E) was presented for the perilipin in EWAT. Values are means ± SEM, *P<0.05 (one-way ANOVA, Bonferroni multiple comparison test). N=8 animals per group, N=2 for histological observations in each group.
2.3.3 **Increased lipolysis in WAT after burn plus LPS is associated with reduced AMPK signaling**

To explore potential mechanisms of increased lipolysis in WAT after burn plus LPS, we explored the activation of HSL and MAPK. Unexpectedly, Western blot analysis showed that lipolysis-related phosphorylation of HSL at Ser563 and Ser660 did not increase in burn or LPS groups and in fact decreased in the burn plus LPS group. The other lipolysis-related phosphorylation of MAPK at Thr202/Tyr204 and Thr185/Tyr187 also decreased in burn, LPS and burn plus LPS groups. These data are consistent with the inhibition of their upstream modulator, PKA (Figure 2.3, A-E). Increased lipolysis is thus attributed to the suppression of inhibitory phosphorylation of HSL at Ser565 as the result of suppression of its upstream regulator AMPKα (Figure 2.4, A-C). Indeed, using well-differentiated 3T3-L1 adipocytes, we confirmed that the stimulation of lipolysis by ER stress and LPS challenge occurs via suppression of AMPKα and HSL phosphorylation at Ser565. We also showed that such lipolysis could be rescued by the AMPK agonist metformin (Figure 2.4, D-F) and AICAR (data not shown).
Figure 2.3  Burn plus LPS do not directly activate HSL and MAPK lipolysis pathway.

Representative images (A) were presented for the Western blot of phosphorylation of HSL at Ser563, Ser660, their upstream modulator of PKA catalyst unit, and MAPK at Thr202/Tyr204 and Thr185/Tyr187 in the adipose tissue. Quantitative densitometric analyses for the above Western blots were presented in (B) to (E). Values are means ± SEM. N=8 animals in each group. *P<0.05 (one-way ANOVA, followed by the Bonferroni post-test).
Figure 2.4  Burn and LPS increased lipolysis by inhibiting AMPK signaling in WAT.

Representative images (A) were presented for the Western blot of phosphorylation of HSL at Ser565 and its upstream modulator of AMPKα in WAT. Quantitative densitometric analyses for Western blots in A were presented in (B) and (C). Values are means ± SEM. N=8 animals in each group. *P<0.05 (one-way ANOVA, Bonferroni post-test). Representative images (D) were presented for the Western blots of perilipin, phospho-AMPKα and phospho-HSL (Ser565) in in vitro differentiated 3T3-L1 adipocytes with or without pre-treatment of 1mM metformin for 6 hours and then challenged by 5μg/ml Tunicamycin and 100ng/ml LPS for 3 or 6 hours, respectively. Quantitative densitometric analyses for Western blots in D were presented in (E) and (F). Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test). In vitro experiments on 3T3-L1 adipocytes were repeated 3 times.
2.3.4 **Burn plus LPS increases adipocyte apoptosis**

To determine if apoptosis was contributing to increased lipolysis after burn plus LPS, we performed TUNEL staining on WAT sections (Figure 2.5, A). Burn and LPS individually and synergistically increased adipocyte apoptosis in WAT (Figure 2.5, B) and this correlated with lipid content of the liver (Figure 2.5, C). We also found that burn plus LPS stimulated pro-apoptotic signals, shown by the significant increase of caspase-9, caspase-3, cleaved caspase-3 and cleaved caspase-7 in WAT (Figure 2.6, A-E).
Figure 2.5  Burn plus LPS promoted apoptosis in WAT: TUNEL staining.

Representative images (A) were presented for the immunofluorescent TUNEL staining of EWAT. Arrows indicate TUNEL-positive cells (magnification x200). Quantitative analysis of positive TUNEL staining cells in EWAT was presented in (B). Values are means ± SEM. P<0.05 in each paired comparison between groups except that between burn and LPS only (one-way ANOVA, Bonferroni post-test). Correlate coefficient analysis between percentage of ORO positive hepatocytes and percentage of TUNEL positive cells in WAT was presented in (C). N=2 in each group.
Figure 2.6  Burn plus LPS promoted apoptosis in WAT: evidence of pro-apoptotic signaling.

Representative images (A) were presented for the Western blots of pro-apoptotic signaling molecules including caspase-9, caspase-3, cleaved-caspase-3 and cleaved caspase-7. Quantitative densitometric analyses for the above Western blots were presented in (B) to (E). Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test). N=8 animals per group.
2.3.5 **Burn and LPS synergistically induce apoptosis in WAT**

Apoptosis is associated with ER stress predominantly through transcription factor CHOP (Han et al., 2013). We found significantly higher expression of Chop mRNA (Figure 2.7, A) and protein (Figure 2.7, B-C) in burn, LPS and burn plus LPS compared to sham. The protein levels of ATF6 and cleaved ATF6 were also significantly higher in burn, LPS and burn plus LPS (Figure 2.7, B, D, and E). CHOP co-localized with the pro-apoptotic marker cleaved caspase-3 in double immunofluorescent staining (Figure 2.7, F and G). While burn alone increased the number of cells with ER stress, LPS predominately increased the apoptotic responses. A synergistic effect was observed in the burn plus LPS group (Figure 2.8, B-F).
Figure 2.7  Burn plus LPS increased ER stress which correlated with apoptosis in WAT.

RT-qPCR analysis of mRNA levels of Chop in EWAT was presented in (A). RT-qPCR data were normalized to 18s rRNA as an internal control. Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test).

Representative images (B) were presented for the Western blots of ER stress markers (CHOP, ATF6) in WAT. Quantitative densitometric analyses for the above Western blots were presented in (C) to (E). Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test). Percentage of immunofluorescent positive cells in WAT in each treatment group was presented in (F). Immunofluorescent double staining of cleaved caspase-3 and CHOP in WAT (magnification, ×400) was presented in (G). N=8 animals per group, N=2 for histological observations in each group.
Figure 2.8  Activation of pro-apoptotic signaling correlates with ER stress in WAT of rats with burn plus LPS.

Correlate coefficient analysis between CHOP positive ER stressed cells and cleaved-caspase 3 positive pro-apoptotic cells in the WAT in burn plus LPS treated animals was presented in (A). Quantitation and statistical analysis of immunofluorescent double staining of adipose tissue by CHOP and cleaved caspase-3 antibodies was presented in (B) to (F). Values are means ± SEM. *P<0.05 (one-way ANOVA, followed by the Bonferroni post-test). N=2 animals in each group. 3 fields are randomly chosen for the cell counting in each animal.
2.3.6 Increased macrophage infiltration and inflammasome activation correlate with apoptosis in WAT

Western blot analyses of NLRP3, caspase-1 and IL-1β in WAT demonstrated that the NLRP3 inflammasome was activated in burn plus LPS animals (Figure 2.9, A to D). Immunofluorescent double staining showed co-localization of the macrophage marker MAC387 (Yu et al., 2010) and pro-apoptotic marker cleaved caspase-3 (Figure 2.9, E and F), suggesting that WAT macrophages were undergoing apoptosis. Burn, LPS and burn plus LPS increased WAT macrophage (MAC387-positive cells) infiltration (Figure 2.10, B), while LPS and burn plus LPS further stimulated macrophage apoptosis (Figure 2.10, C and D). There was also a significant increase in adipocytic (MAC387 negative cells) and overall apoptosis in the burn plus LPS group (Figure 2.10, E and F).
Figure 2.9  Burn and LPS stimulated macrophage infiltration and inflammasome activation which correlated with apoptosis in WAT.

Representative images (A) were presented for the Western blots of NLRP3, caspase-1 and IL-1β in WAT.

Quantitative densitometric analysis for Western blot of NLRP3, caspase-1 and IL-1β in WAT was presented in (B) to (D). Values are means ± SEM. *P<0.05 (one-way ANOVA, Bonferroni post-test). For IL-1β, P value was calculated for 17 kDa mature form. Percentage of immunofluorescent positive cells in WAT in each treatment group was presented in (E). Immunofluorescent double staining of cleaved caspase-3 and MAC387 in WAT was presented in (F) (magnification, x400). N=8 animals per group, N=2 for histological observations in each group.
Figure 2.10  Activation of pro-apoptotic signaling correlates with macrophage infiltration in WAT of rats with burn plus LPS.

Correlate coefficient analysis between MAC387 positive macrophages and cleaved caspase-3 positive pro-apoptotic cells in the WAT in burn plus LPS treated animals was presented in (A). Quantitation and statistical analysis of immunofluorescent double staining of adipose tissue by MAC387 and cleaved caspase-3 antibodies was presented in (B) to (F). Values are means ± SEM. *P<0.05 (one-way ANOVA, followed by the Bonferroni post-test). N=2 animals in each group. 3 fields were randomly chosen for the cell counting in each animal.
2.4 Discussion

Using the rat 2-hit model of severe burn injury followed by LPS intraperitoneal injection, we mimicked the clinical scenario of burn injury followed by septic response and investigated the lipid metabolism cross-talk between WAT and liver during critical illness. We found that there is significant more WAT lipolysis and hepatic fat infiltration in burn plus LPS compared to burn alone. Mechanistically, we attribute the enhanced lipolysis in WAT mainly to the degradation of the perilipin coating of the lipid droplet and to the impairment of AMPK signaling as well as its downstream lipolysis-inhibiting HSL phosphorylation at Ser565. We also found that augmented ER stress, NLRP3 inflammasome activation and apoptosis converged to enhance WAT lipolysis under conditions of severe acute stress.

WAT and liver are in constant communication with one another. For example, JNK1 activation in WAT causes diet-induced hepatic IR (Smith and George, 2009). Moreover, inhibition of hepatic eIF2α involved in protein synthesis and ER stress impairs WAT insulin sensitivity (Birkenfeld et al.). In the current study, we demonstrated that increased WAT apoptosis is positively correlated with lipid infiltration in the liver, implicating increased WAT lipolysis as a source of lipid for deposition in the liver. While this partly explains the morphologic changes in the liver after severe burn, further investigation is needed to determine the mechanisms underlying pronounced hepatic steatosis following thermal injury. It is also possible that decreased fatty acid oxidation and out-bound lipid transportation due to impaired hepatocyte function contribute to increased fat content in the liver.
Interestingly, we did not observe a significant elevation of circulating FFA, glycerol or triglycerides in burn, LPS or burn plus LPS groups (Figure 2.11). There are two possible explanations for this. First, plasma lipid concentration represents an equilibrium between what is released and what is taken up and thus may not reflect the changes in flux (Bradbury, 2006), as is indicated in high fat-fed mice which exhibit no significant increases in blood FFA or glycerol (Baranowski et al., 2008). Secondly, considering the severity of the intervention of burn plus LPS, we did not fast the animals before collecting the blood samples. Differences in food intake of the animals would significantly affect the plasma FFA, glycerol and triglyceride levels. Particularly, we observed significant weight loss in LPS only and burn plus LPS groups but not in burn only group (Figure 2.1, A). The possible explanation could be that the body weight changes under such conditions are closely related with the amount of the food intake. The rats experienced significant decrease of meal size right after burn, their food intake were recovered when sacrificed on post-burn day 4 in burn only group, whereas decrease of food intake in the rats receiving sub-lethal dose of LPS was maximized when they were sacrificed 24 hours after the LPS injection.

Nonetheless, the correlation of TUNEL staining in WAT and ORO staining in liver suggests that increased lipolysis in WAT contributes to the lipid infiltration in the liver. Hepatic steatosis contributes to augmented hepatic ER stress, mitochondrial dysfunction and insulin resistance (Kidani and Bensinger, 2012; Palasciano et al., 2007). Moreover, it may impair the hepatic clearance of LPS (Walley et al., 2014). As shown in the current animal study and in other clinical observations (Grisouard et al., 2012; Szalowska et al., 2011), LPS strongly activates
lipolysis in WAT, which could instigate a vicious positive feedback loop between WAT lipolysis, hepatic lipid accumulation and hepatic LPS clearance.
Figure 2.11  No significant changes are detected in serum level of free fatty acid (A), glycerol (B) or triglyceride (C) among different treatment groups.

Values are means ± SEM. N=8 animals in each group. No significant changes are detected upon one-way ANOVA.
As a mechanism of energy reservation and buffering, lipolysis of WAT is regulated by multiple signaling pathways (Jaworski et al., 2007; Lampidonis et al., 2011). The first step of lipid mobilization from WAT is the phosphorylation or degradation of perilipin and activation of desnutrin/adipose triglyceride lipase (ATGL) which converts triacylglycerol (TAG) to diacylglycerol (DAG). Activated HSL then converts DAG into 2-monoacylglycerol (MAG) which, in turn, is broken down by monoacylglycerol lipase (MGL) into FFA and glycerol. HSL can convert TAG to DAG and has long been considered the key regulator of lipolysis and its activation the driving force for hyperlipidemia since MGL is abundant and its catalyzing activity is not rate-limited (Zechner et al., 2012). This is true in low-grade, chronic adipose stress conditions such as obesity and diabetes, where increased lipolysis occurs in parallel to activation of PKA and HSL phosphorylation at Ser563, Ser660 (Deng et al., 2012). Based on our observations, HSL and its upstream modulators PKA and MAPK were all suppressed under severe and acute traumatic stress conditions, yet we still observed augmented lipolysis and even more severe ectopic lipid deposition in liver. In the current study, we demonstrated that the inhibition of AMPKα and its downstream phosphorylation of HSL at Ser565 contribute to activation of lipolysis in WAT under severe stress conditions.

We also observed increased apoptosis in WAT, which has been implicated in the enhancement of lipolysis (Yasuhara et al., 2006). To address the causative factors of increased apoptosis, we first determined whether there was augmented ER stress in WAT after burn plus LPS since it is well-accepted that ER stress triggers apoptosis under multiple circumstances (Asai et al., 2007; Yasuhara et al., 2006). Here we showed that burn injury alone significantly induces
ER stress in WAT. Together with LPS mediated pro-apoptotic signaling, burn plus LPS further stimulated ER stress and apoptosis, as shown by the robust activation of apoptosis-related ER stress markers CHOP and ATF6 as well as enhanced TUNEL staining. Changes in the pro-apoptotic signaling indicated that ER stress induced by burn injury mainly activated the intrinsic pathway of apoptosis which is manifested by activation of caspase-9 and increased cleavage of caspase-3 (Siegel, 2006). Caspase-7 is a direct substrate of caspase-1, one of the products of inflammasome activation. As such, significantly increased cleavage of caspase-7 in LPS and burn plus LPS groups suggests the contribution of inflammation and inflammasome activation on apoptosis (Lamkanfi and Kanneganti, 2010).

We have previously reported the activation of the inflammasome in the WAT of burn patients (Stanojcic et al., 2014). As the WAT was collected from the wound or adjacent area in these experiments, this raises the question whether inflammasome activation also occurs in distal tissue and organs. In the current study, we observed in experimental animals that severe burn injury and LPS trigger macrophage infiltration and subsequent NLRP3 inflammasome activation in WAT distal to burn wound. It has long been postulated that the damage-associated molecular pattern molecules (DAMPs) contribute to inflammasome activation (Franchi et al., 2009; Martinon et al., 2009). While the identity of the DAMP(s) involved is undetermined to date, augmented ER stress may be responsible for the production of DAMPs considering the concomitance of ER stress and inflammasome activation in WAT (Sasaki and Yoshida, 2015). Furthermore, since the outcome of NLRP3 inflammasome activation is the maturation of pro-inflammatory cytokine IL-1β which then contributes to increased lipolysis, IR and hyperglycemia,
the resulting prolonged hyperglycemic response further enhances and sustains inflammasome activation and pro-inflammatory responses, serving as another positive feedback loop contributing to increased post-burn morbidity and mortality (Benetti et al., 2013; Schroder et al., 2010). Accordingly, multiple therapeutic agents would hold promise to alleviate lipolysis by inhibiting the apoptosis of the adipocytes in WAT, such as α-melanocyte stimulating hormone (αMSH) (Cao et al., 2017) and SNAP23 (Feng et al., 2018).

In summary, in the 2-hit model of burn plus LPS, we observed increased apoptosis and lipolysis in WAT which correlate with adipose ER stress and inflammasome activation. The mechanism responsible for lipolysis following major trauma and sepsis may be related to the inhibition of AMPKα signaling and is distinct from that induced by chronic inflammation. Post-burn WAT lipolysis correlates with fat infiltration in liver and is thus taken as a pre-hepatic pathological change, forming a positive feedback loop which drives the vicious cycle of post-traumatic stress response, hypermetabolism and immunological impairment in severe burn plus sepsis (Figure 2.12).
Figure 2.12  Increased WAT lipolysis and its contribution to immunological and metabolic impairment in the 2-hit rat model of burn plus LPS.

In the acute phase after major trauma, such as an extensive burn, excessive DAMPs and PAMPs, which are derived from wounds, gut, homeostasis derangement and damaged tissues, redistribute to liver and WAT, induce ER stress and stimulate inflammasome activation in these tissues. In the 2-hit model, LPS exacerbates impaired metabolism by stimulating both canonical WAT lipolysis and a pro-inflammatory response in the liver. Lipid deposition in the liver not only augments hepatic ER stress and impairs hepatic metabolic signaling but also inhibits LPS clearance and further stimulates the inflammasome, which perturbs hepatic immune function (A). ER stress and inflammasome activation in WAT contribute to apoptosis and lipolysis, releasing FFA and glycerol which, subsequently, accumulate in the liver (B).
Chapter 3  Hepatic ER Stress, Inflammasome Activation, Liver Dysfunction and Damage

This chapter is adapted from below published original research article:


3.1 Introduction

To investigate the hepatic pathology of stress induced liver dysfunction and liver damage after major burn injury, we conducted experimental study using the same 2-hit rat model of 60% TBSA scald burn plus LPS as in the Chapter 2 and analyzed the hepatic ER stress, mitochondrial dysregulation, hepatic NLRP3 inflammasome activation and demonstrated their contribution to liver dysfunction and damage.

Sepsis and associated multi-organ failure are the major causes of death in extensively burned patients who survive the initial phase of burn shock (Rex, 2012). Large-scale clinical studies showed that sepsis increased intensive care unit resource utilization and mortality in patients with traumatic injury. Despite the reduction in the incidence of sepsis over the last two decades, there has been no reduction in sepsis-associated mortality (Osborn et al., 2004; Wafaisade et al., 2011). Research is thus required to understand the molecular mechanisms of
the pathological changes in burn patients with sepsis and to find out the effective therapeutic targets accordingly.

Sepsis is an infection-induced systemic inflammatory response that has profound impact on metabolism, immunity, and tissue regeneration. Our previous studies have shown that the liver is the central organ for metabolism, immunity and the target organ for stress and inflammation induced parenchymal cell damage in burn (Jeschke, 2009; Jeschke et al., 2007). It has also been shown in microarray analysis that there is a significant albeit temporary up-regulation of the expression of hepatic genes involved in immune response and receptor activity in the animals with endotoxemia (Croner et al., 2009). Furthermore, overwhelming pro-inflammatory cytokines (e.g. Tumor Necrosis Factor α (TNFα), Interleukin-1β (IL-1β), Interleukin-6 (IL-6)) signal septic response via membrane-associated receptors such as cytokine receptors and TLRs (Finnerty et al., 2007; Paterson et al., 2003). In recent years, there is a growing attention to the importance of cytosolic signaling pathways of inflammation. Among these, NOD-like receptor, pyrin domain containing 3 (NLRP3) inflammasome is of particular interest since it can be activated by a number of different stimuli which are common in severe trauma and are termed as damage-associated molecular patterns (DAMPs) (Gross et al., 2011). Especially, our previous study showed that burn injury induces hepatic endoplasmic reticulum (ER) stress and subsequent unfolded protein responses and this is closely correlated with post-burn metabolic dysfunction and insulin resistance (Jeschke et al., 2012). It is thus important to know whether there is interplay between hepatic ER stress, inflammasome activation and
metabolic derangement and how these processes are linked together in severely burned patients.

In a 25% TBSA scald burn murine model, Osuka et al observed that burn injury induced NLRP3 inflammasome activation primarily in macrophages, where it played a protective role in the host response to severe injury (Osuka et al., 2012). The report correlated burn injury with inflammasome activation, but the finding of survival advantage associated with caspase 1 blockade is somehow different from more lethal models of infection or sepsis (Gentile and Moldawer, 2012).

To improve our understanding of inflammasome activation in a more clinically relevant setting, we used a two-hit rat model of major burn plus intraperitoneal injection of sub-lethal dose of *pseudomonas aeruginosa*-derived lipopolysaccharide (LPS). We hypothesized that 1) severe burn injury and following stress responses produce DAMPs that signal NLRP3 inflammasome activation; and 2) this inflammasome activation exacerbates post-burn metabolic dysfunction in the liver.
3.2 Materials and Methods

3.2.1 Animal model

Animal experiments were approved by the Animal Care and Use Committee of Sunnybrook Research Institute in Toronto, Ontario, Canada. The National Institutes of Health Guidelines for the Care and Use of Experimental Animals were met.

Male Sprague Dawley rats (n=6 per group), 275 to 300 grams, were purchased from Charles Rivers Laboratory International Inc. (MA, USA) and were allowed to acclimate for 1 week before we conducted experiments. Rats were housed in the Sunnybrook Research Institute animal care facility and received a high protein diet (Ensure, #22017C8, Abbott Laboratories, IL, USA) and water ad libitum from arrival until sacrifice. Ensure was administered 7 days before the study to adjust the animals to the liquid diet.

A well-established method was used to induce a full-thickness scald burn (Herndon et al., 1978; Jeschke et al., 2011b). The treatment groups included sham, sham + LPS, burn, and burn + LPS. Animals were anesthetized (Ketamine 40mg/kg body weight and Xylazine 5mg/kg body weight, both injected intraperitoneally), the dorsum of the trunk and the abdomen were shaved, and then a 60% TBSA burn was induced by placing the animals in a mold that exposed defined areas of the skin of the back and abdomen. The mold was placed in a 98°C water bath, scalding the back for 10s and the abdomen for 1.5s. Full-thickness cutaneous burn was confirmed by histological section. Lactated Ringer’s solution (40mL/kg body weight) was administered intraperitoneally immediately after the burn for resuscitation. After recovering
from the anesthesia, the rats were placed into separate cages. Sham animals were anesthetized and shaved but not burned.

Animals in the LPS groups (sham + LPS and burn + LPS) received intraperitoneal injection of 10mg/kg *pseudomonas aeruginosa*-derived LPS (Sigma, St Louis, MO, L9143) 72 hours post-burn. Rats were sacrificed 24 hours after the LPS injection (or 96 hours after burn).

### 3.2.2 Plasma and tissue collection

Blood was collected into EDTA-containing tubes (30 μL of 0.5 M EDTA). The tubes were placed on ice temporarily for at least 30 min and centrifuged at 4°C at 1000×g for 10 min, and then the plasma supernatant was aliquoted for later analysis. Liver tissues were collected after brief portal vein perfusion with phosphate-buffered saline (20 mL) and were either immediately frozen in dry ice and then stored at -80°C for further analysis or put in 10% formalin overnight and then transfer to 70% ethanol for paraffin-embedding and tissue slides preparation for immunohistochemical analysis.

### 3.2.3 Real-time quantitative RT-PCR

Total RNA was isolated from liver tissue following manufacturer’s instructions (RNaseasy Mini Kit; Qiagen, Hilden, Germany), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and reverse transcribed (Applied Biosystems, San Diego, CA, USA). Real-time PCR was performed on cDNA with the housekeeping gene rRNA 18S. Target genes included inflammasome activation related genes interleukin-1β (*Il-1*), EGF module-containing Mucin-like hormone Receptor 1 (*Emr*), *Nlrp3*; ER stress marker genes
CCAAT/-enhancer-binding protein homologous protein (*Chop*), 78kDa Glucose-regulated protein/Binding immunoglobulin protein (*Bip*), X-box binding protein 1-spliced form (*Xbp1*-s), Protein disulfide isomerase (*Pdi*); and glucose and lipid metabolic modulator genes Peroxisome proliferator-activated receptor gamma coactivator 1-α (*Pgc 1α*), glucose 6-phosphatase (*G6pase*), fatty acid synthase (*Fasn*), salt inducible kinase 1 (*Sik 1*). The sequences of primers were listed in Table 3.1.

<table>
<thead>
<tr>
<th>Table 3.1 Primer sequences for qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sequence, 5’ to 3’</strong></td>
</tr>
<tr>
<td><strong>Il-1</strong> Forward GCACAGTTCCCCAACTGGTA</td>
</tr>
<tr>
<td>Reverse ACACGGGTTCCATGGTGAAG</td>
</tr>
<tr>
<td><strong>Emr</strong> Forward GCCATAGCCACCTTCTGGT</td>
</tr>
<tr>
<td>Reverse ATAGCGCAAGCTTCTGGTT</td>
</tr>
<tr>
<td><strong>Nlrp3</strong> Forward CAGACCTCCAAGACCACGACTG</td>
</tr>
<tr>
<td>Reverse CATCCGCAGCCAATGAACAGAG</td>
</tr>
<tr>
<td><strong>Chop</strong> Forward AGCGCCTGACCAGGGAGGTA</td>
</tr>
<tr>
<td>Reverse GCTTGGCACCTGCGTGATGGT</td>
</tr>
<tr>
<td><strong>Bip</strong> Forward TCGTCGCTTCTCCTGGCTAC</td>
</tr>
<tr>
<td>Reverse TCATCTTGCCGCGCCTGTGG</td>
</tr>
<tr>
<td><strong>Xbp1-s</strong> Forward GAGTCCGCAGCAGGTG</td>
</tr>
<tr>
<td>Reverse CGTCAGAATCCCATGGGAA</td>
</tr>
<tr>
<td><strong>Pdi</strong> Forward CTGGTCCCGGCCCTCGATT</td>
</tr>
<tr>
<td>Reverse ACGTCTGAGGCGGAGGCGAG</td>
</tr>
<tr>
<td><strong>Pgc-1α</strong> Forward AAAGGGCAAGCAGAGA</td>
</tr>
<tr>
<td>Reverse GTAAATCACACGGCGCTCTT</td>
</tr>
<tr>
<td><strong>G6pase</strong> Forward CCCAGACTAGATCCTGACAGAAT</td>
</tr>
<tr>
<td>Reverse GCACAACGCTCTTTCTTATTACC</td>
</tr>
<tr>
<td><strong>Fasn</strong> Forward CACAGCATTCAGTCTATCCACAGA</td>
</tr>
<tr>
<td>Reverse CACAGCCAACCAGATGCTTTCA</td>
</tr>
<tr>
<td><strong>Sik 1</strong> Forward CGATGGATGCGGCGACCC</td>
</tr>
<tr>
<td>Reverse TGCCAGCACCTGCTCGTGTG</td>
</tr>
</tbody>
</table>
3.2.4 Western blotting

Antibodies against rat phosphorylated AMP-activated protein kinase (phospho-AMPK) α and β, total AMPKα and β, phosphorylated Protein kinase A catalyst unit (phospho-PKA C), SIRT1, Caspase 3 (CASP3) and GAPDH were purchased from Cell Signaling (Danvers, MA, USA). Anti-GRP78/BIP and anti-Peroxisome proliferator-activated receptor (PPAR)-α antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-NLRP3 and anti-PGC-1α antibodies were purchased from EMD Millipore (Billerica, MA, USA). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific Inc. (Rockford, IL, USA).

 Approximately 40 mg of frozen liver tissue was homogenized in 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% (w/v) NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 2.5 mM sodium pyrophosphate, and 1x Complete protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN, USA). The homogenate was centrifuged at 12,000 ×g for 30 min at 4°C and the pellet discarded. Western blotting was performed with 30μg of protein per well. Band intensities were quantified with the Image J software (NIH Bethesda, MD, USA). GAPDH was used as loading control.

3.2.5 Blood glucose level, plasma assay and immunohistochemical analysis for liver damage assessment

Blood glucose level was determined using blood glucose strips (Lifescan Europe, 6300 Zug, Switzerland). Liver damage was assessed by 1) quantifying plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using colorimetric activity assays
(BioVision, Milpitas, CA, USA); 2) immunohistochemical analysis of CASP3 (#9662, Cell Signaling, Danvers, MA, USA) and TUNEL calorimetric assay (G7360, Promega, Madison, WI, USA) were performed according to the product protocol.

### 3.2.6 Statistical analysis

Statistically significant differences were detected by a one-way analysis of variance with student’s t-tests. Data are presented as mean ± SD (n=6 in each group). Significance was accepted at p < 0.05.
3.3 Results

3.3.1 The two-hit of burn injury with LPS injection induces liver damage

We observed liver damage in rats received 2-hit treatment. There was significant elevation of the plasma activity of ALT and AST in the burn + LPS group compared to burn alone or LPS alone, indicating more profound parenchymal liver damage in burn + LPS group (Figure 3.1, A and B). Immunohistochemical study of liver tissue indicated robust elevation of CASP3 in LPS only and burn + LPS group (data not shown). Western blot of CASP3 also showed significant elevation of CASP3 expression in liver tissue in LPS only, burn only and burn plus LPS groups (Figure 3.1, C and D). A focal positive TUNEL staining was found in liver of burn + LPS treatment group (Figure 3.1, E-H). In addition, in the LPS alone and burn + LPS groups, we observed decreased food intake (data not shown) and weight loss (Figure 3.1, I). Despite the reduced food intake and consequent weight loss in these two groups, the blood glucose level remained high at around upper normal range (Figure 3.1, J). These data indicated that burn plus LPS caused liver damage by inducing apoptosis in a subpopulation of hepatocytes which led to increase of ALT and AST in the serum of these animals.
Enzymatic indicators and histological evidence of liver damage, weight loss and changes in blood glucose level in the two-hit rat model of burn and sepsis.

Plasma level of ALT (A) and AST (B) was determined by calorimetric enzyme activity assay. The normal range is 17.5-30.2 U/L for ALT and 45.7-80.8 U/L for AST. Representative images of Western blotting (C) and densitometry analysis (D) of Caspase 3 in liver tissue were presented, normalized by loading control of GAPDH. Immunohistochemical analysis of TUNEL staining of liver tissue was presented in (E) to (H) (400 × magnification). Arrows with and without tails indicated positive and negative staining of TUNEL, respectively. Weight gain/loss (I) was presented in percentage of pre-experimental weight. The level of blood glucose (J) elevated to slightly above normal range (4-6 mM) in burn only group and burn + LPS group (compared with sham, p>0.05). For (A), (B), (D), (I) and (J), data presented are mean ± SD (n=6 in each group), *p<0.05, **p<0.01 vs sham.
3.3.2  *Burn plus LPS injection augment hepatic NLRP3 inflammasome activation*

Burn, LPS, and burn + LPS increased NLRP3 inflammasome activation in the liver. We observed significantly increased *Il-1, Emr, and Nlrp3* mRNA expression (Figure 3.2, A-C) and a significant elevation of NLRP3 protein (Figure 3.2, D and E) in the liver of burn and burn + LPS groups compared to the sham.

**Figure 3.2  Two-hit models of burn plus LPS injection augments inflammasome formation in rat liver.**

IL-1 (A), EMR (B) and NLRP3 (C) mRNA expression in the liver of animals of the sham, sham + LPS, burn, burn + LPS treatment groups (n=6 in each group) was measured by quantitative RT-PCR. Bars showed relative expression of each gene against the 18S house keeping gene. Protein level of NLRP3 in liver was determined by western blot (D) and densitometry analysis (normalized by loading control of GAPDH) (E). Data presented are mean ± SD, *p<0.05, **p<0.01 vs sham.
3.3.3  **Burn and LPS injection induce hepatic ER stress**

We observed significantly increased expression of Chop, Xbp1-s, and Pdi genes, markers of ER stress in liver, in sham + LPS and burn + LPS groups (Figure 3.3, A, C and D). Bip mRNA and BIP protein levels increased significantly in the LPS, burn, and burn + LPS groups (Figure 3.3, B, E and F). Therefore, burn injury, LPS, and their combination led to hepatic ER stress.

![Figure 3.3](image)

**Figure 3.3**  **Burn and LPS induced hepatic ER stress.**

Levels of gene expression of ER stress markers Chop (A), Bip(B), Xbp1-s (C), and Pdi (D) in the liver of animals of the sham, sham + LPS, burn, burn + LPS treatment groups was measured by quantitative RT-PCR. Bars showed relative expression of each gene against the 18S housekeeping gene. Representative images (E) of Western blot and densitometry analysis (F) of BIP in liver tissue was presented, normalized by loading control of GAPDH. Data shown are in mean ± SD (n=6 in each group), *p<0.05, **p<0.01 vs sham.
3.3.4 *Burn induces hepatic metabolic dysfunction*

To determine the underlying mechanism for the differences in weight and glucose, we monitored gene expression of hepatic metabolic modulators, including: *G6pase*, which is the final-step catalyst in gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose level (Hiyama et al., 2013); *Fasn*, which catalyze the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH and thus is fundamental to energy storage and biosynthesis of hormones and other important biological molecules; *Sik 1*, which is a serine-threonine kinase related with steroidogenesis and metabolic regulation in adipose tissue (Okamoto et al., 2004). We also determined the hepatic protein level of G6Pase and SCD1, which are the rate limiting enzymes catalyzing the biosynthesis of monounsaturated fatty acids (Mauvoisin and Mounier, 2011). In the burn group, *G6pase, Fasn, and Sik1* increased significantly compared to sham. In LPS group, *G6pase* mRNA level decreased compared to sham (Figure 3.4, A-C). Protein level of G6Pase in liver tissue decreased significantly in LPS only and burn + LPS groups (Figure 3.4, D and F) whereas level of SCD1 decreased significantly in burn only and burn + LPS group (Figure 3.4, E and G).
Figure 3.4  Burn and LPS induced changes in gene transcription and expression of metabolic modulators in liver.

Levels of gene expression of hepatic metabolic modulator G6Pase (A), Fasn (B), and Sik1 (C) in the animals in sham, sham + LPS, burn, burn + LPS treatment groups was measured by quantitative RT-PCR. Bars showed relative expression of each gene against the 18S housekeeping gene. Representative images of Western blot of G6Pase (D) and SCD1 (E) in liver tissue were presented together with their densitometry analysis (F and G, respectively) normalized by loading control of GAPDH. Data presented are in mean ± SD (n=6 in each group), *p<0.05, **p<0.01 vs sham.
We were particularly interested in the gene expression and tissue abundance of PGC-1α in the liver since it has profound impact on mitochondria energetic metabolism, glucose metabolism, and lipid metabolism (Scarpulla, 2011). We observed increased gene expression of Pgc-1α in all treatment groups (Figure 3.5, A).

3.3.5 Unlike burn which down-regulates SIRT1, LPS inhibits PKA C/AMPKα.

LPS alone caused a significant decrease in the protein level of spliced PGC-1α (Figure 3.5, B-D). To investigate the possible mechanisms of such PGC-1α inhibition, we measured the activation of PKA C, SIRT1 and AMPK, which are upstream regulators of spliced and full-length PGC-1α, respectively. Western blot analysis showed that LPS alone decreased phospho-AMPKα and phospho-PKA C whereas there were no significant changes in phosphor-AMPKβ and PPARα (Figure 3.6, A-G). We found that SIRT1 decreased in burn and burn + LPS groups but not LPS only group (data not shown).
Figure 3.5  Burn and LPS induced changes in PGC 1α in liver.

mRNA level of Pgc-1α in liver (A) was measured by quantitative RT-PCR with 18S housekeeping gene as the reference. Representative images of the Western blot of PGC-1α in liver tissue were presented in (B). PGC-1α has 2 isoforms: 113kD full-length form and 38kD spliced form. Densitometry analysis of the Western blot (C and D) was normalized by loading control of GAPDH. Data presented are in mean ± SD (n=6 in each group), *p<0.05, **p<0.01 vs sham.
Figure 3.6  LPS reduced PGC-1α by inhibiting its upstream regulators.

Representative images of the Western blot of phosphor-PKA C, phosphor- and total- AMPKα and β, as well as PPAR-α were presented in (A). Densitometry analysis of the Western blot (B to G) was normalized by loading control of GAPDH. Data presented are in mean ± SD (n=6 in each group), *p<0.05 vs sham.
3.4 Discussion

In this study, by using a two-hit rat model of burn plus LPS intraperitoneal injection, we investigated the activation of NLRP3 inflammasome and ER stress in liver, their interplay and its impact on post-burn metabolism. We found that both burn and LPS induce significant NLRP3 inflammasome activation in liver and such NLRP3 inflammasome activation augmented liver damage and metabolic derangement in burn plus LPS group. This is the first study to report NLRP3 inflammasome activation in liver tissue after burn injury.

Our previous studies have demonstrated that severe burn injury induces enormous and persisting stress response and hypermetabolism (Jeschke et al., 2011a). The post-burn stress response leads to the accumulation of unfolded and misfolded proteins in the ER and increased intracellular heat shock and chaperone proteins (Bolder et al., 2006). Post-burn hypermetabolism causes increased ATP production, increased OXPHO and ROS production in the mitochondria, and increased gene transcription (Cheung and Jeschke, 2011). All of these increased intracellular biomolecules are DAMPs indicating challenging environment and have been shown to trigger NLRP3 inflammasome activation in other disease models (Mathews et al., 2013; Rotta Detto Loria et al., 2013). Composed of NLRP, ASC, and pro-caspase 1, the NLRP3 inflammasome generally assembles in macrophages where it activates caspase 1 to ultimately produce IL-1β which is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Weber et al., 2010). Moreover, IL-1β interferes with insulin receptor signaling (Choi and Nakahira, 2011) and may thus directly exacerbate metabolic derangement. In the current study, we confirmed such NLRP3 inflammasome activation in the liver in burn and sepsis animals by showing
increased inﬂammasome related gene expression (Il-1, Nlrp3) and increased NLRP3 protein. EMR is a specific marker of macrophages (Khazen et al., 2005). The very similar pattern of Emr mRNA expression with Il-1 and Nlrp3 suggests macrophage involvement of liver inﬂammasome activation. Moreover, our observations suggest that thermal injury with or without sepsis induce NLRP3 inﬂammasome activation in the liver. This is consistent with our understanding that ER stress produces DAMPs and may thus contribute to the initiation of inﬂammasome formation (Menu et al., 2012).

We showed in the current study that ER stress and inﬂammasome activation (assessed by gene expression and protein analysis) shared the same pattern in the LPS, burn, and burn + LPS groups. Further research in knockout models will be important to establish whether there is a causative relationship between these two pathological processes. We noticed that there was less robust ER stress-related genes expression in burn only group. The possible explanation might be that the rats were recovering from initial thermal injury after 96 hours. Our previous study showed that the stress response post-burn peaks indeed between 24-48 hours (Gauglitz et al., 2010). Nevertheless, we did observe augmented responses of both ER stress and inﬂammasome activation in burn + LPS treatment group.

Enhanced ER stress and NLRP3 inﬂammasome activation affected post-burn metabolism. By monitoring the metabolic modulator genes’ expression, liver function, and blood glucose level, we noticed increased metabolic demand from burn injury and lowered metabolic capacity by LPS treatment. Robust increase of G6pase, Fasn, and Sik 1 in burn group postulates an increased need for gluconeogenesis and lipid metabolism whereas decreased protein level of
G6Pase in LPS only and burn plus LPS groups indicated impaired metabolic functioning attributed to LPS treatment. Significant decrease of hepatic SCD1 might be due to insulin resistance of burn and burn plus LPS groups (Stefan et al., 2008).

Particularly, PGC-1α was of our research interest for its multiple, potent roles in mitochondria respiration, gluconeogenesis, lipid metabolism, etc. PGC-1α has two isoforms: a 113 kD full-length isoform and a 38 kD spliced isoform. Spliced PGC-1α has the same functional domain with its full-length form but can more freely transport between the nucleus, mitochondria, ER, and cytosol, and thus has a more powerful effect on metabolic modulation (Shen et al., 2012). A significant increase of Pgc-1α mRNA expression in all three treatment groups suggested increased demand for it. However, in the LPS group, we did not observe corresponding increased protein levels of full-length PGC-1α and actually observed significantly decreased levels of spliced PGC-1α. This may partly account for the metabolic impairment and liver dysfunction in the burn + LPS group.

We previously reported that post-burn ER stress induced hepatic apoptosis contributing to liver damage (Jeschke et al., 2009; Marshall et al., 2013). In the current study, immunohistochemical analysis showed that LPS may augment such post-burn liver damage (Figure 3.1, E-H).

Our results support previously reported Osuka’s finding that macrophages actively involve NLRP3 inflammasome activation. Moreover, our above observation and findings may reasonably explain the controversy of survival advantage of inflammasome activation and its detrimental effects on clinical outcome: burn and LPS induced inflammasome activation has
more profound impact on metabolic impairment and liver dysfunction than its protective effect of cytokines balancing (12). Considering the difference of severity of injury between the two studies (60% TBSA burn with second hit of LPS versus 25% TBSA burn), we may also postulate the different modes of inflammasome activation upon different intensity of stimuli (i.e. being protective upon mild injury and being detrimental upon severe injury).

We did not observe significant elevation of blood glucose level in this animal model. However, considering the unwellness of the animals in the treatment groups which included weight loss (Figure 3.1, I) and decreased food intake (data not shown), blood glucose level of over 7mM was still quite noticeable, which might still be indicative for post-burn hypermetabolism.

To investigate whether LPS, ER stress, and/or inflammasome activation directly or indirectly interfere with PGC-1α expression and function, we examined the upstream modulators of PGC-1α. Three regulatory pathways were studied. The first is PKA C/PGC-1α interaction. It has been shown that activation of PKA can significantly increase the nuclear content of spliced PGC-1α and, thus, PKA modulates PGC-1α-dependent signaling (Chang et al., 2010). We found significantly decreased phospho-PKA C indicating the inhibition of PKA activity in the LPS group, which was consistent with the decreased level of spliced PGC-1α. Therefore, we infer that PKA C inhibition at least partly contributes to attenuation of spliced PGC-1α in burn plus LPS treatment group.

The second regulatory pathway is AMPK. AMPK may activate PGC-1α through phosphorylation of specific serine and threonine residues (Canto and Auwerx, 2009). By
western blotting analysis of AMPK system in this two-hit model, we found that there was a significant decrease in phospho-AMPKα in the burn + LPS group. There were no significant changes in the level of phospho-AMPKβ and total AMPKα and AMPKβ. Since AMPKα is the catalyst unit whilst AMPKβ is regulatory, our result suggests that AMPK activity is directly inhibited at its catalyst unit in burn plus LPS treatment group. The consistency of attenuation of PGC-1α and AMPKα activity may also indicate the possibility of involvement of AMPKα in PGC-1α regulation.

The third upstream regulatory molecule of PGC-1α is SIRT1 which activates PGC-1 through NAD⁺ dependent deacetylation. In this way, SIRT1 links metabolic perturbation with cellular transcriptional output. We observed inhibition of SIRT1 in burn and burn plus LPS treatment group but not LPS only group. Based on this observation, we may reasonably postulate that the inhibition of PGC-1α in burn plus LPS treatment group is the overlay of burn induced SIRT1 inhibition and PKA C/AMPKα signaling blockage mainly induced by LPS. Further confirmative research is needed to establish such causative relationship between PGC-1α inhibition and liver dysfunction and damage.

Western blotting analysis of PPAR-α did not show significant changes among different treatment groups. It is thus unlikely that PPAR-α in liver would respond to the changes in burn or LPS induced stress or inflammasome activation. Since PGC-1α is usually regarded as the co-activator of PPAR-α (Haemmerle et al., 2011), our result may indicate that PGC-1α plays regulatory role in PPAR-α transcriptional activity.
Based on this study we summarize that there is inflammasome activation in the liver after burn and LPS administration. Second, NLRP3 inflammasome activation contributes to the post-burn ER stress response and the two pathological processes exacerbate metabolic dysfunction in the liver. Finally, PGC-1α most likely plays an important role in the hypermetabolic response after burn and may be regulated by PKA C, AMPKα and SIRT1signaling pathways. Future studies will investigate whether PGC-1α, PKA C, AMPKα and SIRT1 may represent potential therapeutic targets for the treatment of post-burn ER stress, inflammasome activation, and subsequent metabolic dysfunction.

In conclusion, burn injury and LPS administration augment hepatic ER stress as compared with any single insult and induce significant NLRP3 inflammasome activation in the liver, exacerbating hepatic metabolic dysfunction and liver damage (Figure 3.7).
Figure 3.7  Hepatic ER stress and NLRP3 inflammasome activation exacerbate hepatic metabolic dysfunction and liver damage in the 2-hit rat model of burn plus LPS.
Chapter 4  Hepatic fat infiltration and liver damage

This chapter is adapted from the below original research article:


4.1 Introduction

To further elucidate the hepatic pathology after major burn injury, we established a mouse model of high fat diet (HFD) induced chronic hepatic stress as a result of obesity in tandem with the acute perturbation of homeostasis upon 20% TBSA scald burn to demonstrate augmented ER stress, mitochondrial impairment, hepatic fat infiltration, cell damage and impaired liver function in HFD plus burn animals, contributing to worse clinical outcomes.

Modern civilization features redundant access to food supply and thus excessive caloric intake which is the leading cause of pandemic obesity (Ginter and Simko, 2012). Consequently, clinicians are seeing more and more obese patients than ever in history. Even though there is an increased incidence of certain comorbid health problems (such as diabetes, hypertension, sleeping dyspnea) in obese people, their health is generally unaffected unless challenged by additional insults such as trauma, infection, etc. Indeed, multiple clinical studies have shown higher morbidity and mortality rates in obese patients upon acute injury or sickness although
the underlying mechanisms are largely unclear (Kraft et al., 2012; Wang, 2014; Yan et al., 2013). Compared to the wealth of knowledge of metabolic derangements in either obesity (i.e. diabetes) (Samuel and Shulman, 2016) or post-trauma (Jeschke et al., 2012), there is a dearth of literature concerning the pathology of severe trauma in the obese population. More interestingly, our previous clinical observations showed that mild obesity (BMI between 30 and 34.9) is beneficial whereas morbid obesity (BMI>40) is detrimental to trauma victims (Jeschke et al., 2013). Hence, there is a fascinating conflict in terms of what is different about mild versus morbid obesity. We therefore asked what the underlying mechanisms by which severe obesity worsens clinical outcomes are.

Since the liver is the central metabolic organ, we hypothesized that the chronic hepatic stress as a result of obesity (Lionetti et al., 2014; Mantena et al., 2009; Wires et al., 2017) augmented the acute perturbation of homeostasis post-burn (Jeschke, 2009; Jeschke and Boehning, 2012) and contributed to worse clinical outcomes in this group of patients.

4.2 Materials and Methods

4.2.1 Animal model

Animal experiments were approved by the Animal Care and Use Committee of Sunnybrook Research Institute (AUP #467) in Toronto, ON. The National Institutes of Health Guidelines for the Care and Use of Experimental Animals were met. 6-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (ME, USA) and were randomly chosen to receive high fat diet (HFD, TD.06414, Harlan Laboratories, WI, USA) to induce obesity; mice
were fed low fat diet (LFD, TD.08806, Harlan Laboratories, WI, USA) as control. After 16 weeks of feeding, intraperitoneal glucose tolerance test (IPGTT) was performed by intraperitoneal injection of 20% glucose solution (2g glucose per kilogram body weight) after overnight fasting followed by blood glucose measurement (Accu-Chek test strips, Roche, USA) at 0, 15, 30, 60 and 120 minutes after the glucose intraperitoneal injection. The animals in each group were sub-divided into sham and burned groups (N=6 in each group). HFD/LFD and water was given ad libitum upon arrival until sacrifice. To evaluate the effects of different diets and burn injury on the pathophysiological changes in mice, the animals were randomized into 4 groups: LFD sham, HFD sham, LFD burn, and HFD burn. A well-established method was used to induce a full-thickness scald burn of 20% TBSA (Jeschke et al., 2011b). Second IPGTT was performed on post-burn day 6 and all the animals were sacrificed on post-burn day 7.

4.2.2 Plasma and tissue collection

Blood was collected from portal vein and cardiac puncture, respectively. Blood and liver tissue were processed as previously described (Diao et al., 2015).

4.2.3 Western blotting

Antibodies against p-ACC (Ser79), ACC, FASN, CPT1A, IP3R1, VDAC, phospho-Akt (Ser473), Rictor, MFN2, BiP, CHOP, caspase-1, cleaved caspase-1, cleaved caspase-3, PARP, and GAPDH were purchased from Cell Signaling (Danvers, MA, USA). Anti- NLRP3, anti-XBP1, and anti-ATF6 antibodies were purchased from EMD Millipore (Billerica, MA, USA). Anti-IP3R3 antibody was purchased from BD Biosciences (San Jose, CA, USA). Clarity Western ECL substrate was purchased from Bio-Rad (Hercules, CA, USA). Liver homogenates (50μg of protein) were separated by 10% SDS-PAGE gel, then transferred to nitrocellulose membrane as previously
described (Diao et al., 2015), and blots were probed using the antibodies listed above. Band intensities were detected, normalized and quantified with the Chemidoc and Image Lab 5.0 software (Bio-Rad Laboratories, Hercules, CA). GAPDH was used as loading control.

4.2.4 In-gel mitochondrial ETC activity assays

In-gel mitochondrial ETC activity assays were performed as described previously (Auger et al., 2017; Wittig et al., 2007).

4.2.5 Immunofluorescent multi-channel staining of liver

Antibody staining was performed as described previously (Amini-Nik et al., 2014; Diao et al., 2015). Primary antibodies were the same as in Western blotting. The percentage of marker-positive cells was determined by taking representative images and directly counting cell number by blindfolded third party. Cell enumerations for each experiment are listed in the text.

4.2.6 Hematoxylin and eosin (H&E) staining and Transmission Electron Microscopy (TEM) of tissue sections

Liver tissue was fixed, sectioned, and stained as described previously. (Amini-Nik et al., 2014; Diao et al., 2015)

4.2.7 Determination of free fatty acids (FFA), glycerol and triglyceride levels in blood

Levels of FFA, glycerol and triglyceride in the blood were determined using FFA, glycerol and triglyceride colorimetric assay kits according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, Michigan, USA).

4.2.8 Statistical analysis
The statistical analysis was performed using Prism version 5.01 (GraphPad Software, San Diego, CA). One-way ANOVA with Bonferroni’s Multiple Comparison Test was used unless otherwise specified and $P < 0.05$ was considered statistically significant.
4.3 Results

4.3.1 HFD and burn lead to hepatic fat infiltration and increased lipolysis

Based on the well accepted murine model of HFD-induced obesity (Jiang et al., 2005), we fed the mice with either HFD or LFD for 16 weeks and we observed significantly higher body weight gain (50% higher on average) in the HFD group versus the LFD group by the end of the 16 weeks of feeding (Figure 4.1, A). If taking LFD mice as with normal BMI of 25, the equivalent BMI of HFD mice is between 35 and 40. Concomitant elevation of blood glucose level was observed in HFD mice (Figure 4.1, B, p<0.05). We also conducted an IPGTT and found the impaired glucose clearance in HFD mice (Figure 4.1, C) confirming metabolic alterations.

We then applied a thermal injury of 20% TBSA (Finnerty et al., 2009) by the end of the 17th week after the initiation of the feeding. We ended the study 6 days after the thermal injury and conducted another IPGTT which showed impaired blood glucose clearance at 30 minutes and 1 hour after the intraperitoneal injection of dextran (Figure 4.1, D and E). These IPGTT results indicate increased insulin resistance in the obese mice both in sham and post-burn.
Figure 4.1  16 weeks of HFD establishes obese mice with insulin resistance.

Weight gains (A), level of blood glucose (B), and IPGTT before the burn injury (C) was compared between mice fed with LFD or HFD. IPGTT was also performed in LFD and HFD mice 6 days after sham burn (D) and burn injury (E). Data are presented as means ± SEM. Statistical analysis was performed using a two-tailed Student’s t-test with *P<0.05 considered statistically significant. N=12 animals per group for (A) to (C), N=6 animals per group for (D) and (E).
Gross examination of the liver at the end of the study revealed some yellow/pink discoloration of the liver in HFD sham and LFD burn animals. This color change was even more pronounced in the HFD burned mice (Figure 4.2, A), indicating increased hepatic fat infiltration; this finding was confirmed by H&E staining which clearly shows increased fat infiltration of the liver in HFD or burned animals, with the greatest fat infiltration in HFD burned animals (Figure 4.2, B).

We hypothesized that increased hepatic fat infiltration is due to increased levels of circulating free fatty acids (FFA). When comparing plasma levels of FFA, we observed elevated FFA in HFD versus LFD mice regardless of the comparison between portal and central vein blood samples or between shams and scald burned animals (Figure 4.2, C, p<0.05). More importantly, we observed significantly higher level of FFA in central vein blood than that in portal vein blood in burned mice (Figure 4.2, D, p<0.05) but not in shams (Figure 4.2, E). Such significantly elevated FFA in central vein blood indicates increased lipolysis of the peripheral adipose tissue after thermal injury (Diao et al., 2015). These observations indicated that a HFD followed by a burn injury is associated with increased lipolysis and circulating FFA, resulting in an increased pre-load of lipids to the liver contributing to substantial hepatic fat infiltration.
Figure 4.2 Augmented hepatic fat infiltration and increased lipolysis and circulating FFA in obese mice after thermal injury.

Representative gross pathology images of the whole livers taken from different treatment groups (A) were presented alongside representative images of H&E staining of mice liver tissue from different groups (B). Plasma level of FFA in blood taken from portal vein and central vein in different treatment groups were compared among LFD versus HFD animals (C). Plasma levels of FFA in blood taken from portal vein versus central vein were also compared in shams (D) and burned mice (E). Data are presented as means ± SEM. Statistical analysis was performed using one way ANOVA followed by Bonferroni’s posthoc test was performed for (C) to (E) with *P<0.05 considered statistically significant and **P<0.01. N=6 animals per group.
4.3.2  *De novo lipogenesis is not activated in HFD mice after thermal injury*

To examine how increased FFA pre-load was associated with fat infiltration of the liver, we hypothesized and determined if there was an increase in *de novo* lipogenesis commonly considered as the significant source of intra-hepatocellular lipids in fatty liver diseases (Solinas et al., 2015). Western blot analysis showed the level of liver tissue inhibitory phospho-acetyl CoA carboxylase (Ser79) (p-ACC) was significantly decreased in HFD fed mice with or without burn injury as compared with LFD sham (Figure 4.3, A and B, p<0.05), implicating increased conversion from acetyl-CoA to malonyl-CoA in HFD fed mice. However, the level of hepatic fatty acid synthase (FASN), the key rate-limiting enzyme of *de novo* lipogenesis (Ameer et al., 2014), was significantly decreased in HFD fed mice, especially in HFD burned group (Figure 4.3, E and F, p<0.01), indicating that there is no activation of lipogenesis albeit the possible increased substrate pressure of malonyl-CoA. There were no significant changes in liver tissue level of p-ACC and FASN in LFD burned mice as compared with LFD shams, indicating that burn alone has no significant impact on hepatic lipogenesis. To further confirm this finding, we performed immunofluorescent staining of p-ACC and FASN in the liver tissue sections (Figure 4.3, C and G). Statistical analysis of the positive cell counts demonstrated results consistent with the Western blot analyses (Figure 4.3, D and H). Hence, despite the increased FFA pre-load, there is no evidence supporting the significant increase in the *de novo* lipogenesis in the liver tissue after HFD and/or thermal injury.
Figure 4.3  Repression of de novo lipogenesis in HFD mice after thermal injury.

Representative images (A) and quantitative densitometric analyses (B) of the Western blot of phospho-ACC (Ser79) and ACC were presented alongside immunofluorescent staining of phospho-ACC (C, magnification ×200) and percentage of phospho-ACC positive cells (D) in liver tissue. Representative images (E) and quantitative densitometric analyses (F) of the Western blot of FASN were presented alongside immunofluorescent staining of FASN (G, magnification ×200) and percentage of FASN positive cells (H) in liver tissue. Data are presented as means ± SEM. *P<0.05 and **P<0.01. N=6 animals per group.
4.3.3 Decreased hepatic lipid $\beta$-oxidation and attenuated mitochondrial electron transport chain (ETC) function associate with hepatic fat infiltration

To investigate the mechanisms of the increased hepatic fat infiltration, we asked next whether it can be attributed to reduced lipid oxidation. We measured the expression of hepatic carnitine palmitoyltransferase-1 (CPT1A) which is the rate-limiting enzyme for liver mitochondrial $\beta$-oxidation, translocating fatty acids across the mitochondrial membrane. The level of the expression of CPT1A would thus reflect the hepatic $\beta$-oxidation activity (Lee et al., 2011).

Western blot analysis showed increased expression of CPT1A in HFD shams and LFD burned mice as compared with LFD sham (Figure 4.4, A and B, p<0.01) but not in HFD burn animals. Such an increase in CPT1A in HFD sham and LFD burn is consistent with other studies showing that hepatic mitochondrial $\beta$-oxidation is enhanced in the liver of genetically obese (or/ob) mice (Brady et al., 1985), HFD fed rats (Mollica et al., 2009) as well as in patients with steatohepatitis (Sanyal et al., 2001), implicating the increased substrate pressure and activation of the compensatory mechanisms of lipid turnover such as hepatic peroxisome proliferator-activated receptor alpha (PPAR$\alpha$). However, there was a significantly lower level of CPT1A in HFD burned animals as compared with that of HFD only or burn only group. It is interesting to note the divergent response of lipid metabolism between HFD sham and HFD burned animals. While there is likely an increase in malonyl-CoA in both groups due to the activation of ACC, it seems that in HFD sham mice significantly activated lipid $\beta$-oxidation might be compensatory enough to limit the magnitude of the fat infiltration whereas in HFD burned mice, the
accumulation of the malonyl-CoA might be overwhelming and significantly inhibit β-oxidation, contributing to augmented hepatic fat infiltration (Lopez-Vinas et al., 2007).

To confirm our Western blotting data we performed immunofluorescent staining of CPT1A in liver tissue sections. We noticed stronger positive signals of CPT1A in HFD shams and LFD burned mice as compared with LFD shams (Figure 4.4, C) and statistical analysis of the positive cell counts demonstrated results consistent with Western blot analyses (Figure 4.4, D). This suggests that in HFD burned animals the liver is not able to β-oxidize the significantly increased inflow of FFA from peripheral lipolysis, resulting in hepatic accumulation of FFA.

To further investigate the underlying mechanisms of impaired hepatic lipid metabolism in HFD burned mice, we analysed the mitochondrial ETC activities in line with the changes in the hepatic mitochondrial lipid β-oxidation (Auger et al., 2017). The increase of complex I and complex III activity in HFD groups implies that there may be an increase in ROS production as these are the primary sites of superoxide formation. Moreover, the lowered activity of ATP synthase in the HFD burned group is particularly detrimental, as it suggests impaired energy formation in this cohort of mice (Figure 4.4, E-J) (Gusdon et al., 2014; Murphy, 2009). Furthermore, a significant decrease of the Complex II activity not only reinforced the likelihood of impaired mitochondrial energy production and increased ROS production but also implicated cell damage in HFD burned animals (Tretter et al., 2016).
Figure 4.4  Impaired hepatic lipid oxidation and mitochondrial electron transport chain (ETC) activities in the obese mice after thermal injury.

Representative images (A) and quantitative densitometric analyses (B) of the Western blot of CPT1A were presented alongside immunofluorescent staining of CPT1A (C, magnification ×200) and percentage of CPT1A positive cells (D) in liver tissue. Representative images (E) of native polyacrylamide gel electrophoresis followed by in-gel activity assays for mitochondrial ETC complexes I, II, III, IV and V in the liver tissue were presented together with the quantitative densitometric analyses for the in-gel blots (F to J). Data are presented as means ± SEM.

*P<0.05 and **P<0.01. N=6 animals per group.
4.3.4  Perturbed inter-organelle Ca\(^{2+}\) homeostasis correlates with decreased ER-mitochondrial contact

Considering the importance of Ca\(^{2+}\) homeostasis in mitochondrial bioenergetics, we sought to investigate whether there were perturbation in ER-mitochondrial Ca\(^{2+}\) homeostasis in HFD and/or burned mice as compared with LFD sham by checking several key regulators of mitochondrial Ca\(^{2+}\) channels including inositol 1,4,5-triphosphate receptor 1 (IP3R1), IP3R3, Voltage-dependent anion channel 1 (VDAC1), p-Akt (Figure 4.5, A) (Griffiths and Rutter, 2009; Rieuisset, 2017). While there were no significant changes in IP3R1 among the groups (Figure 4.5, B, p>0.05), there was a significant decrease of IP3R3 levels in burned mice as compared with LFD sham (Figure 4.5, C, p<0.05). Since IP3R3 is the major channel for Ca\(^{2+}\) efflux from ER to mitochondria under mild stress conditions (Kiviluoto et al., 2013), decreased IP3R3 implicated lower Ca\(^{2+}\) levels in mitochondria due to insufficient Ca\(^{2+}\) influx from the ER. Also, considering that VDAC1 is a multi-functional channel involved in Ca\(^{2+}\) and metabolite transport, energy production and in ER-mitochondria structural and functional association (Shoshan-Barmatz et al., 2017), significantly decreased VDAC1 in HFD sham and LFD burned mice not only indicated the possibility of decrease in mitochondrial mass but also implicated the derangement of Ca\(^{2+}\) transport between ER and mitochondria (Figure 4.5, D, p<0.01). Nevertheless, in HFD burned mice, significantly decreased phospho-Akt (Ser473) and Rictor (Figure 4.5, E and F, p<0.05) indicated impairment of Akt-mTORC2 signaling while it has been well accepted that inhibition of Akt-mTORC2 signaling and subsequent phosphorylation of IP3R in general account for the depletion of Ca\(^{2+}\) from ER to mitochondria, thus triggering cell death pathways (Betz et al., 2013).
More importantly, with the growing appreciation of the ER-mitochondrial axis, we postulated that a decrease of ER-mitochondria contact in HFD and/or burned mice would contribute to the aforementioned perturbed inter-organelle Ca\(^{2+}\) homeostasis and mitochondrial energy production. Since mitofusin 2 (MFN2) is widely accepted as the major regulator of the mitochondria-ER contact (de Brito and Scorrano, 2008; Merkwirth and Langer, 2008), and its coupling molecule, OPA1 is the key regulator of mitochondrial inner membrane fusion and cristae structuring (MacVicar and Langer, 2016), we performed Western blot analysis on the two proteins (Figure 4.5, G). A significant decrease of MFN2 in burned mice indicated decreased ER-mitochondrial contact after burn injury. A further decrease of ER-mitochondrial contact was implicated in HFD burned mice as compared with either HFD sham or LFD burn (Figure 4.5, H, p<0.01). A similar pattern was noted for OPA1 levels (Figure 4.5, I, p<0.01). These findings in Western blot analysis were confirmed via transmission electron microscopy (TEM) (Figure 4.6, A-D). As compared with sham, increased space among the ER and mitochondria (arrows) as well as the shrinkage of the mitochondrial cristae (arrow heads) were evident in the liver tissue sections of the burned mice.
Figure 4.5  Mitochondrial metabolic dysfunction is correlated with the perturbed inter-organelle Ca^{2+} homeostasis and mitochondrial dynamics in the liver of obese mice after thermal injury.

Representative images (A) were presented with the quantitative densitometric analyses (B to F) of Western blots for IP3R1, 3, VDAC1, phospho-Akt (Ser473), and Rictor in the liver tissue. Representative images (G) and quantitative densitometric analyses (H and I) of Western blots for MFN2 and OPA1 implicate the changes in mitochondrial dynamics. Data are presented as means ± SEM. *P<0.05 and **P<0.01. N=6 animals per group.
Figure 4.6  The decrease of hepatic ER-mitochondrial contact and mitochondrial structural changes after burn injury.

Representative transmission electron microscope images demonstrate ultrastructural changes in hepatocytes after burn injury. Nu marks nucleolus. Arrows indicate the space between mitochondria and ER; arrow heads point to mitochondrial cristae structure.
4.3.5 Augmented hepatic ER stress, inflammasome activation and aggravated cell damage in HFD mice after thermal injury

With the above evidence of the changes in ER-mitochondrial structure, function and contact, it was reasonable to further postulate a concomitant cellular stress response in HFD and/or burned animals (Shinde et al., 2016). We thus determined the level of several important hepatic ER stress and subsequent unfolded protein responses (UPR) markers (Huang et al., 2017). Western blots (Figure 4.7, A) and densitometric analysis demonstrated augmented ER stress in the liver of HFD and/or burned animals. We observed two patterns of UPR upon the HFD and/or burn injury: 1) the significant activation of hepatic BiP and XBP-1 in either HFD sham or LFD burn group (p<0.01), whereas less significant (BiP) or no significant change (XBP-1) in HFD plus burn group as compared with LFD sham control (Figure 4.7, B and C); 2) the level of ATF6 and CHOP increased significantly in HFD plus burn group (Figure 4.7, D-F, p<0.01).

Considering that CHOP is a pro-apoptotic transcription factor, we then sought for the evidence of cell damage upon HFD and/or thermal injury (Szegezdi et al., 2006). TUNEL staining of liver tissue sections confirmed the aggravated apoptosis in HFD sham, LFD burn and HFD burn groups as compared with LFD sham (Figure 4.8, A). Interestingly, we noticed that hepatocyte apoptosis was more severe in burned mice (Figure 4.8, B, sham vs burn, p<0.01) whereas stromal cell apoptosis was more significant in HFD treatment (Figure 4.8, C, LFD vs HFD, p<0.01). Nevertheless, a significantly higher rate of apoptosis was seen in HFD burned mice when compared to LFD shams and either intervention of HFD or burn alone (p<0.01).
Figure 4.7  Augmented hepatic ER stress in HFD burned mice.

Representative images (A) were presented with the quantitative densitometric analyses (B to F) of Western blot for ER stress markers of BiP, XBP-1, ATF6, and CHOP in liver tissue. Data are presented as means ± SEM. *P<0.05 and **P<0.01. N=6 animals per group.
Figure 4.8  TUNEL staining of liver tissue demonstrated increased liver cell apoptosis in HFD burned mice.

TUNEL and DAPI staining of the liver tissue (A) were presented together with the percentage of TUNEL positive hepatocytes and stromal cells (B and C). Arrows indicate apoptotic hepatocytes and arrowheads indicate apoptotic stromal cells (magnification: ×400 in the upper panel, ×200 in the lower panel).

Data are presented as means ± SEM. *P<0.05 and **P<0.01. N=6 animals per group.
We postulated the increased inflammasome activation which contributes to the apoptosis of the stromal cells (Wen et al., 2011). This was confirmed by the Western blot of NLRP3 and Caspase 1 (both total and cleaved form) in the liver tissue (Figure 4.9, A to D, p<0.01). Also, elevated levels of cleaved Caspase 3 and Poly-ADP ribose polymerase (PARP) were also consistent with the TUNEL staining, demonstrating the aggravated cell damage particularly in HFD burned mice (Figure 4.9, E, p<0.01; F, p<0.05).
Figure 4.9  NLRP3 inflammasome activation, enhanced pro-apoptotic signaling and DNA damage in HFD burned mice.

Representative images (A) were presented with the quantitative densitometric analyses of Western blot for NLRP3, caspase 1, cleaved caspase 1, cleaved caspase 3 and PARP in the liver tissue (B to F).

Data are presented as means ± SEM. *P<0.05 and **P<0.01. N=6 animals per group.
4.4 Discussion

In the current study, we aimed to determine why patients with obesity have altered metabolism and decreased survival after burn. We focused on liver pathology and used a mouse model of HFD induced morbid obesity and 20% TBSA scald burn. The thermal injury model is well described, but we ensured adequate hypermetabolic and inflammatory responses when combining it with high fat diet induced obesity.

In this model, we found profound hepatic fat infiltration in HFD burned mice, which is mainly attributable to increased lipolysis and impaired hepatic lipid β-oxidation and correlates with liver damage. It is very interesting to note that increased hepatic infiltration is not due to increase de novo lipogenesis. To investigate the underlying mechanisms of such metabolic impairment and tissue damage, we compared the hepatic ER stress responses, changes in mitochondrial ETC activities, ER-mitochondria communication, inflammasome activation and apoptosis signaling among sham, burn and HFD plus burn groups. We noticed that with a burn injury alone the hepatic responses are generally including the activation of ER UPR of increased expression of BiP and XPB-1 (Ozcan et al., 2004; Taylor and Dillin, 2013), and higher levels of CPT1A for increased β-oxidation. Mitochondrial ETC activities and ATP synthesis are also well maintained and inflammasome activation and apoptosis are mildly increased. Nevertheless, at least two phenomena implicated metabolic derangement after burn injury as compared with sham animals: 1) decreased mitochondria mass and perturbation of mitochondrial Ca²⁺ homeostasis as is indicated by the changes in IP3R3 and VDAC1; 2) decreased ER-mitochondria contact and mitochondrial dynamics as is manifested by the changes in the levels of MFN2 and
OPA1 and morphological alterations seen in TEM. With the onset of ER stress, to chaperone the increased nascent, unfolded or misfolded proteins, BiP tends to dissociate from IP3R1 which is the major channel of Ca\textsuperscript{2+} flux from ER to mitochondria under physiological conditions, leading to inhibition of the IP3R1 Ca\textsuperscript{2+} channel. Moreover, a significant decrease of the IP3R3 level as seen in burned animals may also have an impact by decreasing mitochondrial Ca\textsuperscript{2+} levels. This is consistent with the decreased ATP synthase activity, decreased ER-mitochondria contact, and therefore, decreased energy production and, possibly concomitant increase in ROS production (Wang et al., 2017). Furthermore, derangement of mitochondrial dynamics as was manifested by the changes in MFN2 and OPA1 links impaired energy production with cell damage (MacVicar and Langer, 2016).

In HFD induced obese mice after burn injury, drastic changes in almost every aforementioned cellular process brought about a significantly different outcome as compared with burn injury alone. Augmented ER stress was beyond the cellular capacity of molecular chaperoning and pro-apoptotic signaling was activated as was indicated by significantly increased levels of CHOP. In contrast to the inhibition of Ca\textsuperscript{2+} efflux from hepatic ER to mitochondria in LFD burned mice, there might be dysfunctional Ca\textsuperscript{2+} transport from ER to mitochondria due to significant activation of IP3R which is termed IP3-induced Ca\textsuperscript{2+} release (Li et al., 2009). We hypothesize that increased mitochondrial Ca\textsuperscript{2+} levels contribute to further impairment of energy production and aggravated cell damage (Hayashi and Su, 2007). Importantly, a greater decrease of ER-mitochondrial communication in HFD burned mice was seen with the concomitant and significant suppression of OPA1 and inhibition of mitochondrial
ETC complexes activities, indicating impairment of mitochondrial energy production, and ultimately, aggravated cell damage (Friedman and Nunnari, 2014).

In summary, in LFD mice, burn injury stimulates an ER stress response with the increased likelihood of a lower level of mitochondrial Ca\(^{2+}\) and subsequent decreased ATP and increased ROS production, as well as decreased ER-mitochondrial contact, all correlated with a mild lipid infiltration. In HFD shams, there is mitochondrial fragmentation and lipid accumulation in the liver albeit compensatory increase of lipid β-oxidation. This is accompanied with NLRP3 inflammasome activation and increased apoptosis as compared with LFD shams. When HFD mice were challenged by burn injury, augmented ER stress induced pro-apoptotic signaling; mitochondrial energy production was significantly impaired with further perturbed Ca\(^{2+}\) homeostasis; uncompensated impairment of ER-mitochondrial contact, faulty β-oxidation, and inflammasome activation occurred, leading to significantly decreased lipid turnover. Subsequent fat infiltration would aggravate ER stress, forming a vicious cycle and leading to liver organ damage (Figure 4.10, A). As such, in animals with obesity and severe burn, it is the impairment of multiple cellular processes which compound upon each other that deregulates lipid homeostasis and, as a consequence, worsens outcomes for obese trauma victims (Figure 4.10, B). Accordingly, early and effective interventions to attenuate ER stress, inflammasome activation, and ROS production, as well as treatment to stimulate mitochondrial dynamics and restore mitochondrial Ca\(^{2+}\) homeostasis would be beneficial to this group of patients.
Figure 4.10  Hepatic fat infiltration is attributable to the vicious cycle of ER stress, mitochondrial dysregulation and cell damage in HFD burned mice.

Hepatic ER stress and disturbed ER-mitochondria communication lead to the derangement of mitochondrial ETC activities, energy production, and impaired lipid metabolism (A). This contributes to the increased hepatic fat infiltration, which, together with hepatic inflammasome activation, results in increased hepatic metabolic dysfunction and liver damage in HFD burned mice (B).
Chapter 5  Stress induces periportal ductal progenitor cells proliferation, contributing to prolonged pro-inflammatory response and hypermetabolism

This chapter is adapted from the below submitted research article:

Diao L, Yousuf Y, Amini-Nik S, Jeschke MG: Increased proliferation of hepatic periportal ductal progenitor cells contributes to persistent hypermetabolism after trauma. JCI insight [submitted]

5.1  Introduction

Severe trauma as major burn injury is always accompanied by acute perturbation of homeostasis and physiological stress response (Jeschke and Boehning, 2012; Jeschke et al., 2012; Long et al., 1979). Multiple clinical studies demonstrated that there are profound and prolonged pro-inflammatory response and hypermetabolic response after major burn injury, contributing to significant morbidity and mortality (Jeschke et al., 2004; Jeschke et al., 2011a). The underlying mechanisms of how the severe trauma leads to prolonged hypermetabolism are still not clearly elucidated. Epigenetic reconfiguration of certain signaling pathways, such as mitochondrial biogenesis and dynamics, and metabolic plasticity of certain cell types, such as muscle cells and adipocytes, are postulated to be the possible driver of such persisting pathophysiology (Abdullahi and Jeschke, 2016; Kanherkar et al., 2014; Porter et al., 2016).

Since the liver is the central metabolic organ and integrating pro-inflammatory signals with the metabolic mediators (Jeschke, 2009), considering its ability and plasticity of continuous self-regeneration (Huch and Dolle, 2016), we hypothesized that the changes in hepatocytes
proliferation and regeneration under stress conditions contribute to prolonged hyperinflammation states and hypermetabolism.

Although it has been well accepted that the liver is a regenerable organ and up to 2/3 of the loss of the liver parenchyma can be recovered by regeneration without jeopardizing the viability of the entire organism (Michalopoulos, 2007), it is still controversy how such a regeneration happens including whether there is single or multiple sources of stem cells, what the triggers of the liver regeneration are and how the liver regeneration is regulated (Tarlow et al., 2014b; Yanger et al., 2014; Yovchev et al., 2008).

In the experiment in Chapter 4, when examining the immunofluorescent staining of p-ACC (Ser79), FASN, and CPT1A, I noticed different patterns of the expression of the key enzymes of lipid metabolism in the hepatocytes between portal triads and central venule systems. In HFD sham animals, while the elevated substrate pressure of malonyl-CoA in central venule zone was mainly attributable to increased conversion of malonyl-CoA from acetyl-CoA which was indicated by the decrease of the inhibitory phosphorylation of ACC at Ser79 (Figure 5.1, A and B), the increased substrate pressure in portal venule zone was attributable to decreased de novo lipogenesis indicated by significant inhibition of the expression of FASN (Figure 5.1, C and D). Also, significant activation of β-oxidation was seen in hepatocytes around portal venule but not central venule in HFD sham group (Figure 5.1, E and F). In LFD fed burned mice, there was a trend of shifting of substrate production for lipogenesis and β-oxidation from portal venule zone to central venule zone (Figure 5.1, B, D, and F). Although both hepatic
lipogenesis and β-oxidation were impaired in HFD burned mice, the underlying mechanisms were different between the hepatocytes of portal triads and central venule systems.

Figure 5.1  Different patterns of the expression of the key enzymes of lipid metabolism between the hepatocytes around portal triads and central venule systems.

Representative images of immunofluorescent staining of phospho-ACC(Ser79) (A, magnification ×200), FASN (C, magnification ×200), and CPT1A (E, magnification ×200) and percentage of phospho-ACC (Ser79)(B), FASN (D) and CPT1A (F) positive cells in portal or central venule systems of liver tissue.

**Zone 1:** portal triad; **Zone 3:** central venule system.

Data are presented as means ± SEM. *P<0.05. N=6 animals per group.
Since the portal triads are where the facultative regeneration of hepatic parenchyma occurs under liver damage and stress conditions (Font-Burgada et al., 2015), considering the long-existing and evidence-supported streaming liver theory (Hoehme et al., 2010; Turner et al., 2011) that the regeneration and maturation of hepatocytes starts from the portal venule, proceeds across the liver plates and ends with apoptosis in the central venule, we hypothesized that liver regeneration under profound stress condition was dominated by proliferation and differentiation of periportal ductal progenitor cells (PDPC) which are bi-potential progenitor cells that can give rise to either hepatocytes or cholangiocytes (Cardinale et al., 2012) whereas liver regeneration under physiological or mild stressful conditions was dominated by self-duplication of mature hepatocytes (Yanger et al., 2014). Moreover, we speculated that hepatocytes regenerated under stress conditions after major burn injury might possess aberrant and persistent inflammatory and/or metabolic profiles and thus contribute to prolonged pro-inflammatory states and hypermetabolism that are commonly seen in major burned patients (Jeschke et al., 2004; Jeschke et al., 2011a).

To test this hypothesis, we generated a Sox9 CreERT2:ROSA26 EYFP reporter mice to trace the PDPC (Kawaguchi, 2013; Kopp et al., 2011) and sought to investigate if there is increased proliferation of this group of cells after burn injury and how they contribute to post-burn pathological changes.
5.2 Materials and Methods

5.2.1 Animal model

Animal experiments were approved by the Animal Care and Use Committee of Sunnybrook Research Institute (AUP #579) in Toronto, ON. The National Institutes of Health Guidelines for the Care and Use of Experimental Animals were met.

Tg(Sox9-crestERT2)1Msan/J mouse (hemizygous, +/-) was purchased from the Jackson Laboratory (Bar Harbor, ME, USA, Stock No. 018829). The mouse was bred to B6.129X1-Gt(Rosa)26Sortm1(EYFP)Cos/J mouse (homozygous, +/+, Bar harbor, ME, USA, Stock No. 006148) to generate Sox9-crestERT2 +/-:ROSA26 EYFP +/- offspring (F1). F1 mice were cross-bred and F2 of Sox9-crestERT2 +/-: ROSA26 EYFP +/- were selected for continuous breeding. Genotyping was performed following the protocol on the official website of the Jackson Laboratory and the primers are listed in Table 5.1.

Table 5.1 Primers for genotyping

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence 5' --&gt; 3'</th>
<th>Primer Type</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox9 Cre</td>
<td>oliMR1084</td>
<td>GCG GTC TGG CAG TAA AAA CTA TC</td>
<td>Transgene</td>
<td>100bp</td>
</tr>
<tr>
<td></td>
<td>oliMR1085</td>
<td>GTG AAA CAG CAT TGC TGT CAC TT</td>
<td>Transgene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oliMR7338</td>
<td>CTA GGC CAC AGA ATT GAA AGA TCT</td>
<td>Internal Positive Control Forward</td>
<td>324bp</td>
</tr>
<tr>
<td></td>
<td>oliMR7339</td>
<td>GTA GGT GGA AAT TCT AGC ATC ATC C</td>
<td>Internal Positive Control Reverse</td>
<td></td>
</tr>
<tr>
<td>EYFP</td>
<td>oliMR4982</td>
<td>AAG ACC GCG AAG AGT TTG TC</td>
<td>Mutant</td>
<td>320bp</td>
</tr>
<tr>
<td></td>
<td>oliMR8545</td>
<td>AAA GTC GCT CTG AGT TGT TAT</td>
<td>Common (both bands: heterozygote)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oliMR8546</td>
<td>GGA GCG GGA GAA ATG GAT ATG</td>
<td>Wild type</td>
<td>600bp</td>
</tr>
</tbody>
</table>
8-11 week-old male mice with the genetic background of Sox9-cre/ERT2\(^{+/−}\): ROSA26 EYFP\(^{+/−}\) were included for the animal experiments. Tamoxifen (Sigma, St Louis, MO) was dissolved at 20mg/ml in corn oil (Sigma) and administered subcutaneously at a dosage of 100mg/kg body weight. Tamoxifen was administered once daily for 3 consecutive days. Considering the potential pharmaceutical effects of tamoxifen/estrogen receptor activation in the hepatic inflammatory response and liver damage (Hsieh et al., 2007; Shimizu et al., 2007), wild type mice of the same age and non-tamoxifen control were also kept for baseline determination. The mice were randomly divided into sham and burned groups and received 30% total body surface area (TBSA) scald burn (Auger et al., 2017) or sham treatment immediately after the first injection of Tamoxifen.

The mice were sacrificed on post-burn day 2, 7, 14, 21, 28, and 42 (referred to as different observational groups). N=6 for each group including sham control. Mice sacrificed on post-burn day 2 received 2 doses of tamoxifen injection.

5.2.2 Liver tissue collection and digestion

Upon sacrificing, the inferior vena cava was cut and the whole liver was collected after brief portal vein perfusion with PBS (2 mL). The liver was weighed and 2 small pieces of liver were taken and frozen immediately on dry ice and then stored at -80°C for gene expression and Western blot analyses. Another piece of liver tissue was fixed in 10% buffered formalin at 4°C overnight, transferred to 70% ethanol and then paraffin embedded for histology. The rest part of the liver tissue was chopped into fine particles less than 1mm\(^3\) and transferred to 5mL
digestion cocktail (200U dispase, 270mg Type I collagenase in 100ml DMEM with 1% Ab/Am) for cell staining and flow cytometry analysis and cell sorting.

5.2.3 Reagents and antibodies

Antibodies against CHOP, phospho-eIF2α, eIF2α, ATF4, BiP, HSP90, CPT1A, PARP, IL-1β, phospho-p38 MAPK, p38 MAPK, GAPDH, and EpCAM (VU1D9) Mouse mAb (Alexa Fluor® 647 Conjugate) were purchased from Cell Signaling (Danvers, MA, USA). Anti-phospho-IRE1α antibody was purchased from Thermo Scientific Inc. (Rockford, IL, USA). Anti-LXRα and anti-GFP antibodies were purchased from Abcam (Cambridge, MA, USA). Clarity Western ECL substrate was purchased from Bio-Rad (Hercules, CA, USA).

5.2.4 Western blotting

Liver homogenate lysates (50μg of protein per well) were separated by 10% SDS-PAGE gel, proteins were transferred to nitrocellulose membrane as previously described (Diao et al., 2015), and then blots were probed using the antibodies listed above. Band intensities were detected, normalized and quantified with the Chemidoc and Image Lab 5.0 software (Bio-Rad Laboratories, Hercules, CA). GAPDH was used as loading control.

5.2.5 Cell staining and flow cytometry

Cells were incubated in digestion cocktail in 37°C for 40 minutes, then added equal volume of DMEM with 10% FBS and filtered through 40μm strainer to a new tube. The cells were then washed with FCM buffer (0.5% BSA in HBSS). Cell count was performed with trypan
blue using TC20™ automatic cell counter (Bio-Rad Laboratories, Hercules, CA). 5 million cells (viability is between 30-50%) were transferred to flow tube and washed with FCM buffer. The cells were then blocked with CD16/32 on ice for 10 minutes followed by incubation with antibodies against EpCAM in FCM buffer on ice for 30 minutes. The sample was then washed once with FCM buffer and then top up with FCM buffer with DAPI. For flow cytometry analysis, all samples were run on the BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA, USA). 100,000 events were collected for each sample and data was analyzed using FlowJo (v. 8.7) software. Cell sorting was run on the BD FACSAriaIIu (BD Biosciences, San Jose, CA, USA).

5.2.6 Immunofluorescent multi-channel staining of liver

Antibody staining was performed as described previously (Amini-Nik et al., 2014; Diao et al., 2015). Primary antibodies were the same as in Western blotting. The percentage of marker-positive cells was determined by taking representative images and directly counting cell number by blindfolded third party. Cell enumerations for each experiment are listed in the text or figure legends.

5.2.7 Microarray transcriptomic analysis

Sorted cells from 4.2.5 were centrifuged and the pellet was dissolved in Trizol and total RNA was extracted using a QIAGEN kit according to the manufacturer’s instructions, and expression profiles were compared using microarray analysis. For gene profile analysis, RNA quality was assessed with a Bioanalyzer (Agilent Technologies), and samples with an RNA integrity number (RIN) greater than 8.0 were included for array. cDNA was generated using
Affymetrix WT-Pico Kit and hybridized onto the Affymetrix Mouse Gene 2.0 ST chips. Analysis of gene expression was performed using Parktec Genotyping Suite for gene level differential expression analysis and Ingenuity Systems Software for canonical signaling pathway analysis. Filter criteria for positive signals are folder changes greater than 2 and ANOVA p-value <0.05.

5.2.8 Statistical analysis

The statistical analysis was performed using Prism version 5.01 (GraphPad Software, San Diego, CA). One-way ANOVA with Bonferroni’s Multiple Comparison Test was used unless otherwise specified and P < 0.05 was considered statistically significant.
5.3 Results

5.3.1 The proliferation of PDPCs increases, contributing to hepatomegaly after major burn injury

To specifically track the proliferation of the PDPCs in the liver after thermal injury, we generated Sox9-cre/ERT2:ROSA26 EYFP mice in which the expression of EYFP in PDPCs is inducible upon tamoxifen treatment. We optimized the protocol for the tamoxifen treatment by comparing different dosage of tamoxifen treatment with either intraperitoneal or subcutaneous injection. Flow cytometry analysis clearly demonstrated that subcutaneous injection of the tamoxifen for 3 consecutive days induced most consistent expression of EYFP (Figure 5s.1), which is consistent with the immunofluorescent staining of the liver tissue sections against anti-GFP antibody (data not shown).

By initiating the tamoxifen injection at the time when the mice were subjected to 30% TBSA scald burn, we were able to trace all the EYFP+ cells as the PDPCs proliferating and differentiating after the thermal injury. When harvesting the cells, we performed cell staining of Alexa Fluor® 647 Conjugated anti-EpCAM (VU1D9) mouse monoclonal antibody to distinguish between PDPCs and PDPC-derived hepatocytes for the flow cytometry study (Figure 5.2, A).

We observed significantly increased cell population of the PDPCs and PDPC-derived hepatocytes (total EYFP+ cells) in PBD7, 14 and 21 as compared with sham (Figure 5.2, B and C), peaking at around PBD14. Together with the significant increase of the EYFP+/EpCAM+ cells in PBD7, 14 and 21 (Figure 5.2, D), this indicated the increased proliferation and differentiation of PDPCs after thermal injury.
To test if the proliferation of PDPCs contributes to the increase of the hepatic parenchyma, we measured the weight of the whole liver of the mice and compared with the body weight of the mice when sacrificed. Since the body weight of the mice was generally stable during the whole observation period (Figure 5s.2), the concomitant significant increase of liver/body weight ratio around PBD14 and 21 indicated hepatomegaly in this period of time (Figure 5.2, E). Interestingly, while the proliferation of PDPCs peaked around PBD14 and significantly attenuated afterwards (Figure 5.2, C), the increase of the liver mass peaked around PBD21 (Figure 5.2, E). This implicates other mechanisms of liver regeneration in addition to the PDPCs proliferation after PBD14.

We also performed immunofluorescent staining of the liver tissue section with anti-GFP antibody to examine the distribution of the EYFP+ cells in the liver (Figure 5.3, A-H). It was clearly demonstrated the increase of the EYFP+ cells around the portal triads after thermal injury. The statistical analysis of the positive cell counts showed consistent results with the flow cytometry analysis (Figure 5.3, I). However, we were unable to see the typical pattern of streaming of liver regeneration from portal triads to central venule systems in this group of EYFP+ cells. Neither were we able to see any significant long term structural changes of the liver after burn injury although temporary hepatic fibrotic changes has been observed in our separate study, which is mainly attributable to the changes in the pathology of myeloid cells (Amini-Nik et al., 2018).
Figure 5.2  Increased proliferation of the PDPCs contributes to hepatomegaly after thermal injury.

Based on gating (A) of hepatocytes of wild type mice, non-tamoxifen treated control, and hepatocytes isolated from Sox9-cre/ERT2^+/^-ROSA26 EYFP^+/+ mice 7 days post-burn, 3 groups of cells were separated: EYFP^+ (P8), EpCAM^-EYFP^+ (P6), and EpCAM^-EYFP^+ (P7). P5 represented EYFP^+ cells which are PDPCs in total including progenitor cells (P7) and progenitor cell derived hepatocytes (P6). Representative spectrum images (B) and statistical analysis of P5 versus total hepatocytes (C) and P7 versus P5 (D) of different groups were presented together with the comparison of liver/body weight ratio among the groups (E).

Data are presented as means ± SEM. **P<0.01 versus all other groups. # P<0.05 versus sham.$ P<0.05 versus PBD42. N=6 animals per group (non-tamoxifen control, sham, and each time point post-burn).
Figure 5.3  Increased proliferation of the PDPCs is around portal venule after thermal injury.

Representative images of the immunofluorescent staining of GFP (A-H, magnification ×100) in different groups were presented together with the statistical analysis of the positive cell counts (I). Dotted circles depict central venule.

Data are presented as means ± SEM. *P<0.05 versus all other groups. N=6 animals per group (non-tamoxifen control, sham, and each time point post-burn).
Figure 5s.1  Optimization of the tamoxifen treatment protocol: dosage and route of administration.

Flow cytometry analysis of the hepatocytes isolated from mice treated with different dosage of tamoxifen (100mg/kg body weight, for 1, 2 or 3 consecutive days) and by either intraperitoneal (IP) or subcutaneous (SC) injection as compared with hepatocytes isolated from non-tamoxifen treated mice (NC). N=3 in each group. *P<0.05, **P<0.01.

Figure 5s.2  The changes in body weight in mice before and after burn injury.

The body weight of all the burned mice included in the study was monitored daily within 2 weeks post-burn, every 3 days from PBD14 to PBD28 and weekly afterwards to the end point. The body weight of the mice decreased slightly in the first 3 days post-burn, restored to pre-burn level within PBD7, kept stable until PBD14, and then slightly increased at an average rate of 70mg/day.
5.3.2 The hepatic stress response correlates with the increased proliferation of PDPCs after major burn injury

We then sought to investigate if hepatic stress response correlated with the PDPCs proliferation after burn injury. We examined the expression of multiple cellular stress markers including phospho-IRE1α, CHOP, phospho-eIF2α versus eIF2α, ATF4, BiP and HSP90 in the liver tissue by Western blotting (Figure 5.4, A) of whole liver lysate samples and densitometry analysis showed that significant hepatic stress response occurred from PBD2 to around PBD21 and resolved after PBD28 to almost normal at PBD42, the end point of our observation (Figure 5.4, B-G). The chronological consistency between the hepatic stress response and PDPCs proliferation implicated their correlation after burn injury. Furthermore, we performed immunofluorescent double staining of the liver sections against anti-HSP90 and anti-GFP antibodies and we found significant co-localization of the HSP90⁺ and GFP⁺ cells in the liver after burn injury (Figure 5.5, A). On PBD2, 7 and 14, almost all the GFP⁺ cells are also HSP90⁺ (Figure 5.5, B and C). This strongly suggested the correlation between hepatic cellular stress and PDPCs proliferation.
Figure 5.4  Hepatic cellular stress response after thermal injury.

Representative images of Western blot (A) was presented together with the densitometric analysis of multiple cellular stress markers including phospho-IRE1α (B), CHOP (C), phospho-eIF2α/eIF2α (D), ATF4 (E), BiP (F) and HSP90 (G).

Data are presented as means ± SEM. * P<0.05 as compared with all the other groups. # P<0.05 as compared with Sham. $ P<0.05 as compared with PBD42. ▽ P<0.05 as compared with PBD21, 28 and 42. N=6 animals per group including non-tamoxifen control, sham and different time point post-burn.
Figure 5.5 Hepatic cellular stress response correlates with PDPCs proliferation after thermal injury.

Representative images of immunofluorescent double staining of HSP90 and GFP of liver tissue sections (A, magnification ×200) were presented together with the statistical analysis of the positive cell counts (B and C).
5.3.3 Increased proliferation of PDPC-derived hepatocytes contributes to persistent pro-inflammatory and hypermetabolism after major burn injury

We next asked whether and how the increased proliferation of the PDPCs contributes to the persistent pro-inflammatory response and hypermetabolism after burn injury. We first performed microarray analysis to compare the transcriptome of 1) PDPCs and PDPC-derived hepatocytes before and 7 days after burn injury; and 2) PDPCs and PDPC-derived hepatocytes versus mature hepatocytes on PBD7 (Table 5.2).

<table>
<thead>
<tr>
<th>Table 5.2 Microarray samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYFP+</td>
</tr>
<tr>
<td>Sham</td>
</tr>
<tr>
<td>PBD7</td>
</tr>
</tbody>
</table>

By setting up the filter criteria as fold changes (linear) <-2 or >2; and ANOVA p-value (condition pair) <0.05, we found that, among the 34472 genes analyzed, 418 genes are differentially expressed between the EYFP+ cells of sham and mice of PBD7 group; and 2344 genes are differentially expressed between the EYFP+ (PDPCs and their progeny) and EYFP- (mature hepatocytes) cells from the same liver of the mice of PBD7 group (data not shown).

We then conducted canonical signaling pathway analysis to unravel how these differentially expressed genes implicate the changes in cell physiology.

There are 5 up-regulated signaling pathways and 11 down-regulated signaling pathways in the EYFP+ cells on PBD7 as compared with that of sham (Table 5.3). When compared the EYFP+ cells with the EYFP- cells on PBD7, we found 52 relevant up-regulated signaling pathways and 12 relevant down-regulated signaling pathways (Table 5.4). Among these, acute phase
response signaling, IL-6 signaling, and p38 MAPK signaling are pathways of note since the activation of these pathways in PDPCs and PDPC-derived hepatocytes after burn injury was not only significantly demonstrated as compared with that of sham (Figure 5.6) but also more robust as compared with mature hepatocytes under the same condition (Figure 5.7). Of special importance, the specific and significant down-regulation of the LXR/RXR signaling pathway was seen in the PDPCs and PDPC-derived hepatocytes post-burn as compared with sham and with the mature hepatocytes at the same time point post-burn (Figure 5.8), implicating the impairment of hepatic lipid homeostasis and overwhelming of pro-inflammatory response in the liver as the result of the increased proliferation and differentiation of the PDPCs (Hong and Tontonoz, 2014). Taking into the consideration of the prevailing concept of immunometabolism as the interaction between the immunological response and metabolism (Hotamisligil, 2017), it is also reasonable to speculate the contribution of such increased proliferation of PDPCs to the persistent hypermetabolism after major burn injury.

Table 5.3 Comparison of the changes in canonical signaling pathways in EYFP⁺ cells in mice of Sham versus PBD7 group

<table>
<thead>
<tr>
<th>Up-regulated signaling pathways</th>
<th>Down-regulated signaling pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>acute phase response signaling</strong></td>
<td><strong>LXR/RXR activation</strong></td>
</tr>
<tr>
<td>coagulation system</td>
<td>production of NO and ROS in macrophages</td>
</tr>
<tr>
<td>type 1 diabetes mellitus signaling</td>
<td>role of NFAT in regulation of immune response</td>
</tr>
<tr>
<td><strong>IL-6 signaling</strong></td>
<td>B cell receptor signaling</td>
</tr>
<tr>
<td><strong>p38 MAPK signaling</strong></td>
<td>PI3K signaling in B lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Th1 pathway</td>
</tr>
<tr>
<td></td>
<td>role of pattern recognition receptors in recognition of bacterial and virus</td>
</tr>
<tr>
<td></td>
<td>FcγRIIb signaling in B lymphocytes</td>
</tr>
<tr>
<td></td>
<td>calcium-induced T lymphocytes apoptosis</td>
</tr>
<tr>
<td></td>
<td>phospholipase C signaling</td>
</tr>
<tr>
<td></td>
<td>p70S6K signaling</td>
</tr>
</tbody>
</table>
Table 5.4  Comparison of the changes in canonical signaling pathways in EYFP\(^{+}\) versus EYFP\(^{-}\) cells in mice of PBD7 group

<table>
<thead>
<tr>
<th>Up-regulated signaling pathways</th>
<th>Down-regulated signaling pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 signaling</td>
<td><strong>LXR/RXR activation</strong></td>
</tr>
<tr>
<td>TREM1 signaling</td>
<td>role of NFAT in regulation of immune response</td>
</tr>
<tr>
<td>Pattern recognition receptors in recognition of bacterial and virus</td>
<td>Th1 pathway</td>
</tr>
<tr>
<td>NF-κB signaling</td>
<td>Th2 pathway</td>
</tr>
<tr>
<td>Toll-like receptor signaling</td>
<td>phospholipase C signaling</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus signaling</td>
<td>PPAR signaling</td>
</tr>
<tr>
<td><strong>Acute phase response signaling</strong></td>
<td>EIF2 signaling</td>
</tr>
<tr>
<td>Coagulation system</td>
<td>Telomerase signaling</td>
</tr>
<tr>
<td><strong>IL-6 signaling</strong></td>
<td>IL-2 signaling</td>
</tr>
<tr>
<td>Cholecystokinin/Gastrin-mediated Signaling Pathway</td>
<td>PTEN signaling</td>
</tr>
<tr>
<td>Cytotoxic lymphocyte mediated apoptosis</td>
<td>PI3K/AKT signaling</td>
</tr>
<tr>
<td>HMGB1 signaling</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td><strong>P38 MAPK signaling</strong></td>
<td></td>
</tr>
<tr>
<td>Tec kinase signaling</td>
<td></td>
</tr>
<tr>
<td>Inteigrin signaling</td>
<td></td>
</tr>
<tr>
<td>Autophagy</td>
<td></td>
</tr>
<tr>
<td>HGF signaling</td>
<td></td>
</tr>
<tr>
<td>MEF2 mediated oxidative stress responses</td>
<td></td>
</tr>
<tr>
<td>PEDF signaling</td>
<td></td>
</tr>
<tr>
<td>inflammasome pathway</td>
<td></td>
</tr>
<tr>
<td>LPS/IL-1 mediated inhibition of RXR function</td>
<td></td>
</tr>
<tr>
<td>Apoptosis signaling</td>
<td></td>
</tr>
<tr>
<td>Fas signaling</td>
<td></td>
</tr>
<tr>
<td>LPS-activated MAPK signaling</td>
<td></td>
</tr>
<tr>
<td>GM-CSF signaling</td>
<td></td>
</tr>
<tr>
<td>VEGF signaling</td>
<td></td>
</tr>
<tr>
<td>Stat3 pathway</td>
<td></td>
</tr>
<tr>
<td>NOS signaling</td>
<td></td>
</tr>
<tr>
<td>Cdc-42 signaling</td>
<td></td>
</tr>
<tr>
<td>ILK signaling</td>
<td></td>
</tr>
<tr>
<td>p53 signaling</td>
<td></td>
</tr>
<tr>
<td>Death receptor signaling</td>
<td></td>
</tr>
<tr>
<td>endothelin 1 signaling</td>
<td></td>
</tr>
<tr>
<td>CXCR4 signaling</td>
<td></td>
</tr>
<tr>
<td>phospholipase C signaling</td>
<td></td>
</tr>
<tr>
<td>p70S6K signaling</td>
<td></td>
</tr>
<tr>
<td>mTOR signaling</td>
<td></td>
</tr>
<tr>
<td>VDR/RXR activation</td>
<td></td>
</tr>
<tr>
<td>notch signaling</td>
<td></td>
</tr>
<tr>
<td>cAMP mediated signaling</td>
<td></td>
</tr>
<tr>
<td>TGF-beta signaling</td>
<td></td>
</tr>
<tr>
<td>IL-10 signaling</td>
<td></td>
</tr>
<tr>
<td>IL-22 signaling</td>
<td></td>
</tr>
<tr>
<td>phagosome formation</td>
<td></td>
</tr>
<tr>
<td>CD40 signaling</td>
<td></td>
</tr>
<tr>
<td>SAPK/ERK signaling</td>
<td></td>
</tr>
<tr>
<td>JAK/Stat signaling</td>
<td></td>
</tr>
<tr>
<td>UVB-induced MAPK signaling</td>
<td></td>
</tr>
<tr>
<td>ERK/MAPK signaling</td>
<td></td>
</tr>
<tr>
<td>VDR/RXR activation signaling</td>
<td></td>
</tr>
<tr>
<td>CNTF signaling</td>
<td></td>
</tr>
<tr>
<td>ErbB2-ErbB3 signaling</td>
<td></td>
</tr>
<tr>
<td>TNFR1 signaling</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.6  Up-regulation of the acute phase response (A), p38 MAPK (B), and IL-6 (C) signaling pathways in PDPCs after thermal injury.

The upper panels were the gene expression patterns when the signaling pathway was activated. The lower panels represented fold changes of the genes in the mice on PBD7 versus sham (p<0.05). N=3 animals per group.
Figure 5.7  On PBD7, acute phase response (A), p38 MAPK (B), and IL-6(C) signaling pathways are more activated in PDPCs as compared with that in mature hepatocytes.

The upper panels were the gene expression patterns when the signaling pathway was activated. The lower panels represented fold changes of the genes in the EYFP⁺ versus EYFP⁻ cells (p<0.05). N=3 animals per group.
Figure 5.8  LXR/RXR signaling pathway is significantly down-regulated in PDPCs postburn.

LXR/RXR signaling pathway of PDPCs is significantly down-regulated after thermal injury (A). LXR/RXR signaling pathway of PDPCs is significantly inhibited as compared with that of mature hepatocytes (B). N=3 in each group.
To confirm our above findings of the activation or inhibition of the signaling pathways and their correlation with the post-burn pro-inflammatory response and hypermetabolism, we examined the levels of the expression of some key modulators or effectors in the liver tissue including LXRα, IL-1β, phospho-p38 MAPK, p38 MAPK, CPT1A and PARP (Figure 5.9, A). The decreased levels of expression of LXRα, as well as the increased levels of expression of pro-IL-1β, matured IL-1β, and phospho-p38 MAPK, were all chronologically consistent with the microarray transcriptomic data.

Specifically, the level of hepatic LXRα significantly decreased between PBD2 to PBD14 with the concomitant presence of the cellular stress response (Figure 5.3) and then significantly increased around PBD21 and PBD28 along with the resolving of the inflammation and the restoration of the homeostasis after the major burn injury (Figure 5.9, B). We observed rapid and significant increase of the expression of hepatic pro-IL-1β from PBD2 to PBD21, resolving to the level of sham animals on PBD28 (Figure 5.9, C). There seemed to be a delayed and more temporal increase of the level of hepatic matured IL-1β which peaked around PBD14 (Figure 5.9, D). Similarly, the level of the hepatic phospho-p38 MAPK versus total p38 MAPK increased significantly around PBD7 to PBD14 (Figure 5.9, E) which, together with the changes in the expression of hepatic LXRα and IL-1β, implicated the activation of the immunological responses and inflammatory signaling between PBD2 to PBD14 or 21.

Moreover, we measured the level of expression of CPT1A, which is the rate-limiting regulator of hepatic β-oxidation (Lee et al., 2011), and it was demonstrated that there was increased β-oxidation from PBD2 to PBD21, reflecting the increased energy demand and
expenditure after burn injury (Figure 5.9, F). We also examined the level expression of PARP in the liver tissue (Figure 5.9, G and H). The significant increase of the level of PARP around PBD2 suggested liver cell damage and increased apoptosis in early post-burn period. Interestingly, we observed a bi-phasic increase of cleaved hepatic PARP post-burn. The second phase of the increase of cleaved PARP was around PBD21, which is concomitant with the attenuation of the PDPCs proliferation from PBD14 to PBD21.

Overall, comparison of the transcriptomics between 1) the PDPCs and PDPC-derived hepatocytes before and 7 days after burn and 2) the PDPCs and mature hepatocytes from the mice 7 days post-burn, together with the analysis of the changes in the expression levels of some key molecules, revealed the significant activation of immunological response and inflammatory signaling in PDPCs and their progeny, contributing to hepatic pro-inflammatory responses and metabolic perturbation after major burn injury.
Figure 5.9  Up-regulated hepatic acute phase response and p38 MAPK signaling followed the decrease of LXRα expression and correlated with increased lipid oxidation and cell damage in the liver after thermal injury.

Representative images of the Western blot (A) and densitometric analysis of the expression of LXRα (B), pro-IL-1β (C) and matured IL-1β (D), phospho-p38 MAPK/p38 MAPK ratio (E), CPT1A (F), and full and cleaved form of PARP (G and H) in the liver tissue.

Data are presented as means ± SEM. * P<0.05 as compared with all the other groups. # P<0.05 as compared with Sham. $ P<0.05 as compared with PBD28. N=6 animals per group including non-tamoxifen control, sham and different time point post-burn.
5.4 Discussion

In the current study, using the reporter mice strain of Sox9-cre/ERT2:ROSA26-EYFP, we were able to lineage-trace the proliferation and differentiation of PDPCs after burn injury. By flow cytometry analysis, we observed increased proliferation of PDPCs which peaks around two weeks post-burn. The pool of progenitors also expands temporarily from one to three weeks post-burn which is concomitant with the increased proliferation and differentiation of PDPCs.

It has been well-accepted that severe liver damage and the impairment of the renewal of hepatic parenchyma by self-duplication of mature hepatocytes trigger the proliferation of the PDPCs for the liver regeneration (Miyajima et al., 2014). Here we have demonstrated that 30% TBSA scald burn is an insult strong enough to activate PDPCs proliferation. By further investigating the chronological feature of hepatic cellular stress response after burn injury, we would suggest the correlation of cellular stress response and the activation of the proliferation of the PDPCs. This correlation was also supported by immunofluorescent double staining of liver tissue sections with antibodies against GFP (to label EYFP$^+$ cells) and HSP90 (cellular stress marker). Furthermore, the increased number of GFP$^+$/HSP90$^+$ cells around portal venule not only implicates that cellular stress response contributes to the activation of the proliferation of the PDPCs but may also suggest that the PDPCs are more vulnerable to stress stimuli as compared with the mature hepatocytes (van Galen et al., 2014). Nevertheless, whether and how the stress signals trigger the proliferation and differentiation of the PDPCs still warrants further investigation.
Two phenomena implicate that increased proliferation of PDPCs may not be the only contributing factor for the hepatomegaly after burn injury:

1. The proliferation of the PDPCs post-burn peaks around PBD14 with the EYFP$^+$ cell population over 20% of the total hepatocytes count. The EYFP$^+$ cell population of PBD7 and PBD21 are both around 15%. However, the liver/body weight ratio peaks around PBD21 within the context of stable or slightly increased body weight. Cells other than PDPCs contribute to the increased liver mass from PBD14 to PBD21.

2. When examining the histological pattern of the liver regeneration after burn injury, we did not see the typical streaming of the hepatocytes from portal triads to central venule systems. We found most of the EYFP$^+$ cells are along the portal venule from PBD2 to PBD7, disseminating to the liver plates around PBD14 and PBD21, but seldom stretching out to the central venule system afterwards.

To better explain the above phenomena, we speculate that the liver regeneration in the early post-burn period is mainly via the proliferation of PDPCs when there is significant cellular stress response and liver damage, whereas two to three weeks after injury, with the approaching of the wound closure and the restoration of total body homeostasis, the cellular stress response is attenuating and the liver regeneration is gradually taken over by the self-renewal of the mature hepatocytes (Tanimizu and Mitaka, 2014).

Moreover, when we determined the hepatic PARP level after burn injury, we noticed a bi-phasic increase of the expression of cleaved form of PARP around both PBD2 and PBD21,
indicating increased apoptosis at these two time points post-burn. It is clear that the first phase of the increase correlates with acute stress response after burn injury which is consistent with the increased expression of the multiple cellular stress markers (Jeschke et al., 2009; Marshall et al., 2013). The second phase of the increase of the cleaved PARP is concomitant with the decrease of the EYFP⁺ cells from around 25% on PBD14 to 15% on PBD21 and 10% on PBD28, implicating the clearance of the PDPCs and PDPC-derived hepatocytes when the homeostasis is finally restored after the injury.

Transcriptomic analysis in the current study reveals the significant activation of pro-inflammatory signaling pathways, including acute phase response signaling pathway, IL-6 signaling pathway, p38 MAPK signaling pathway, in PDPCs and their progeny after burn injury as compared with either the same group of cells before the injury or the mature hepatocytes at the same time point after burn injury. We demonstrated the supportive evidence of the activation of such pro-inflammatory signaling pathways by examining the expression of the key regulators or effectors of the pathways in the liver after burn injury, including the pro-IL1β and its matured form, as well as total and phospho-p38 MAPK.

Recently, there is an increased appreciation of envisioning immune mediators, such as those significantly activated cytokines of TNFα, IL-1β, and IL-6 in PDPCs post-burn, as metabolic hormones (Hotamisligil, 2017) which stimulate metabolic activities by increasing the energy expenditure and substrate consumption (Porter et al., 2016). It is well accepted that the p38 MAPK signaling pathway can be activated by a wide range of cellular stress signals and is critical for immune and immunological responses (Cuenda and Rousseau, 2007). Also, it is evident the
synergistic interactions among these signaling pathways (Yang et al., 2008). Taking together, it is appropriate to consider the expanded population of PDPCs and the activation of the above signaling pathways in these cells as the contributing factor of inflammatory response and hypermetabolism after major burn injury.

Based on our observation, the duration of the pro-inflammatory response and metabolic derangement in the burned mice is around 3 to 4 weeks, peaking at around 2 weeks post-burn. Since the maturation rate of the mice aged 1 to 6 month is about 45 times of that of human (Flurkey et al., 2007), three weeks in mice could be roughly equivalent to 2 years in human. The duration of the pro-inflammatory response and metabolic derangement we observed in this burned mice study is thus consistent with the clinical observations of the persistent pro-inflammatory states and hypermetabolism in major burned patients.

We found the down-regulation of the hepatic LXR/RXR signaling pathway concomitant with the activation of the above pro-inflammatory pathways after burn injury by both the transcriptomic analysis and determination of the changes in the level of expression of LXRα in the liver tissue. It is interesting to notice that, on the one hand, LXR signaling is inhibitory to inflammatory responses and thus the down-regulation of the LXR signaling pathway at least correlates with, if not contributes to, the activation of the pro-inflammatory responses; on the other hand, LXR signaling is pivotal to lipid homeostasis, and especially cholesterol metabolism, in mammals and the repression of the LXR signaling implicates impaired lipid/cholesterol metabolism after burn injury (Kidani and Bensinger, 2012). This finding provides additional
mechanistic explanation to the abnormal lipid metabolism we have seen in the studies in the Chapter 2 and Chapter 4.

Furthermore, since cholesterol is the precursor to all steroid hormones, a constant supply must be available to the adrenal gland. This raises the concern of the importance of the inter-organ crosstalk between liver and adrenal gland under stress conditions such as severe trauma of major burn injury. Although acute stress response is regulated by the hypothalamic-pituitary-adrenal axis, hepatic LXR signaling pathway works as the regulator of the level of substrates for such a response. In the acute stress response, the immediate need for cholesterol substrate is accomplished by the rapid mobilization of intracellular cholesterol stores whereas under chronic stress sustained import of cholesterol into the cell and mitochondria is warranted, and eventually, at the end of the stress response, the flux of adrenal cholesterol must be switched back to storage and efflux (Cummins et al., 2006).

More importantly, this may suggest a novel therapeutic target for the care of the major burn patients early after the injury. It will be interesting to see if early application of LXR agonist to the major burn patients can be beneficial to the control of overwhelming stress response and pro-inflammatory response, as well as the amelioration of the metabolic derangement. Especially, it might be of clinical significance to observe the replacement or synergistic effects of LXR agonist with β-blocker which has been widely used and proved to be effective in the care of major burn patients. There have been several LXR agonists in different phases of clinical trials for the treatment of atherosclerosis. However, a major issue of concern is their undesirable effects on hepatic lipogenesis and thus the increased risk of hepatic steatosis if they are used
for long time (Hong and Tontonoz, 2014). We are curious to see if short term administration of these LXR agonists to major burn patients would be safe and feasible. The potential candidate drugs may include BMS-779788 (also known as XL-652) for which the Phase I clinical trial has been completed (ClinicalTrials.gov identifier: NCT00836602) and GW6340 which has been tested in the animal experiments to act selectively in the intestine and promote reverse cholesterol transport from macrophages (Yasuda et al., 2010).

In conclusion, hepatic cellular stress responses and cell damage induces proliferation and differentiation of PDPCs with activated pro-inflammatory signaling, contributing to the persistent pro-inflammatory response and hypermetabolism after major burn injury (Figure 5.10). LXRα agonists stimulate LXR signaling pathway which is inhibitory to the pro-inflammatory pathways and may thus have potential therapeutic effects to ameliorate the pro-inflammatory response and hypermetabolism if administered early after the burn injury.
Aberrant liver regeneration contributes to persistent pro-inflammatory response and hypermetabolism after major burn injury.

Hepatic cellular stress response and liver damage stimulate liver regeneration from facultative liver stem cells, giving rise to hepatocytes with activated pro-inflammatory and metabolic stressful signaling, contributing to hepatomegaly and persistent pro-inflammatory response and hypermetabolism after major burn injury.
Chapter 6  Thesis summary and future directions

6.1  General discussion

Major burn injury represents the most severe form of trauma and exemplifies acute perturbation of homeostasis and profound stress response followed by persistent immunological and metabolic derangement (Jeschke et al., 2008a; Jeschke et al., 2011a). The drastic pathophysiology contributes to increased incidence of sepsis, multiple organ dysfunction and failure, and mortality (Jeschke et al., 2015). Better therapeutic strategy is thus warranted to improve the restoration of the homeostasis, to alleviate the magnitude and duration of the stress response, and to ameliorate the prolonged pro-inflammatory responses and metabolic derangement.

Based on our previous studies demonstrating that the liver is the functional hub integrating pro-inflammatory signals with the metabolic mediators (Jeschke, 2009), and that significant hepatomegaly, liver dysfunction and liver damage occur after major burn injury (Jeschke et al., 2001; Jeschke et al., 2007), I conducted animal experiments and in vitro studies to investigate the pathological changes in the liver after thermal injury, using different model systems to mimic the clinical scenarios of significant perturbation of homeostasis, profound stress responses, pro-inflammatory responses, and metabolic disorders which are commonly seen in major burned patients. Such a series of mechanistic studies of liver pathology after thermal injury would hold the promise to develop effective interventions for persistent hypermetabolism which, as the consequence of increased levels of systemic pro-inflammatory
cytokines, acute phase proteins, catecholamines, and cortisol, features significantly increased REE, decreased lean body mass and body fat, thus is detrimental to the outcome of the major burned patients (Jeschke et al., 2011a).

6.1.1 Rodent animal models for translational research

Three rodent animal models have been used:

1. Two-hit rat model of 60% TBSA scald burn plus LPS intraperitoneal injection (10mg/kg body weight, administered 3 days post-burn). The rats were young (8-10 weeks). The animals were sacrificed 4 days post-burn. The observation was thus made to understand the sub-acute response after injury (Chapter 2 and 3);

2. HFD induced obesity in mice (16 weeks of HFD with LFD as control) plus 20% TBSA scald burn injury. The mice were middle-aged adults (25 weeks). The animals were sacrificed 7 days post-burn. The observation was made to investigate the pathological changes when the mice were recovered mostly from the acute phase stress of thermal injury (Chapter 4);

3. 30% TBSA scald burn injury in Sox9-cre/ERT2: ROSA26 EYFP mice (tamoxifen was administered subcutaneously at a dosage of 100mg/kg body weight for 3 consecutive days beginning at the time when the mice were burned). The mice were young (8-11 weeks) when the experiments were initiated. The time course study was performed to investigate the pathological changes from 2 days to 6 weeks post-burn (Chapter 5).
We only included male mice and rats in the animal studies mainly for two reasons. First, previous clinical studies have shown that severe thermal injury leads to decreased anabolic hormones over a prolonged period of time and female patients had significantly increased levels of anabolic hormones, which are associated with decreased pro-inflammatory mediators and hypermetabolism (Jeschke et al., 2005; Jeschke et al., 2008b). The exclusion of female mice would help us to better observe the impact of burn injury on the alteration of metabolism by reducing the above divergence of the responses of the animals toward injuries. Second, considering that severe burn injury results in significant changes in hemodynamics, coagulation, metabolism and function of multiple organs and systems, divergence of menstrual cycles of the female mice and rats should also be avoided to maintain a consistent physiological background of the experimental animals.

Another major issue of concern of the study design of the animal experiments with rodents is to optimize the animal model so that the injury incurred is severe enough to demonstrate the distinguishable pathological changes as compared with control and yet, an acceptable mortality rate of the animals could be maintained. This seems to be challenging for two reasons. Firstly, rodents usually manifest the “all or none” phenomena in response to the burn injury. That is, there is a very narrow window between too minor an injury (if the burn size is not big enough) to induce significant pathological changes and too severe an injury to make the animal moribund in a short time. Indeed, we always conducted preliminary animal experiments to define the intensity of the injuries and insults so as to limit the mortality rate of the animals in the experiments to below 10% (data not shown). Secondly, due to the
distinguishable anatomy of the subcutaneous muscle layer in the rodents, the healing of the
wounds is significantly faster than that we observed in human (Abdullahi et al., 2014). For
example, while it is lethal if a 30% TBSA full-thickness burn injury is left untreated in human,
burn wounds of the same size were usually closed spontaneously in mice within 4 to 5 weeks
(animal model in Chapter 5, data not shown). By using the two-hit model of burn plus LPS or
inducing burn injury in morbidly obese mice, we impose multiple harmful insults to the animals
to keep a sub-lethal effect of any of them but induce significant perturbation of the
homeostasis which impacts consequent pathophysiological changes. Accordingly, based on the
preliminary animal experiments, we determined that a 60% TBSA scald burn in rats and 30%
TBSA scald burn in mice could be taken as optimized severity of injury to study the subsequent
pathological changes. However, in the study of burn injury in HFD versus LFD mice, we finally
normalized the area of burn to 20% TBSA with the consideration that these mice were much
larger in size and the fixed area of the mold for the scald burn can only cover a relatively
smaller percentage of the total body surface area. We will further address this issue in the
following part of ‘Limitation of the current study’.

Furthermore, it should always be kept in mind the peculiarity of the post-burn
pathology and prudence should be applied when translating the knowledge we gained in the
animal study with scald burn injury to other forms of trauma such as hemorrhagic shock, blunt
trauma, etc. (Al-Tarrah et al., 2017).
6.1.2 Immunometabolic disorder after trauma: what we can learn from metaflammation

In the Chapter 3 and Chapter 4, in both the rat two-hit model of burn plus LPS and mouse model of HFD plus burn, we demonstrated common pathology in the liver after major burn injury, including hepatic ER stress, inflammasome activation, mitochondrial dysregulation, and liver cell damage. Our data thus implicates an immunometabolic disorder with the integration of the immune response/inflammatory response and metabolic derangement after burn injury. In recent years, there is a rapid growth of the body of the literature in metaflammation which is defined as low-grade, chronic inflammation orchestrated by metabolic cells in response to excess nutrients and energy (Gregor and Hotamisligil, 2011). It is interesting to compare the similarity and difference between the two pathological phenomena. Indeed, both immunometabolic disorders after severe trauma and metaflammation feature hepatic ER stress response, insulin resistance (Jeschke and Boehning, 2012), inflammasome activation (Winkler and Rosen-Wolff, 2015), and aberrant inter-organ cross talk on lipid signaling and metabolism (Abdullahi and Jeschke, 2016; Ertunc and Hotamisligil, 2016). There are also significant differences between the two (Table 6.1) (Hotamisligil, 2017; Ni Choileain et al., 2006).
Table 6.1 Difference between the immunometabolic disorders after severe trauma and metaflammation

<table>
<thead>
<tr>
<th></th>
<th>Immunometabolic disorders after severe trauma</th>
<th>Metaflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>Acute and self-limiting</td>
<td>Chronic and persistent</td>
</tr>
<tr>
<td>Magnitude</td>
<td>Strong</td>
<td>Low-grade</td>
</tr>
<tr>
<td>Innate immune cells</td>
<td>Macrophage and neutrophil</td>
<td>Macrophage</td>
</tr>
<tr>
<td>involved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adaptive immunity</td>
<td>Increased T&lt;sub&gt;reg&lt;/sub&gt; activity</td>
<td>Increased CD4&lt;sup&gt;+&lt;/sup&gt; and CD8&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>involved</td>
<td></td>
<td>Deficient in T&lt;sub&gt;reg&lt;/sub&gt;</td>
</tr>
<tr>
<td>Pathogenic factor(s)</td>
<td>Multiple (hypoxia, ROS, toxins, infection, etc)</td>
<td>excess nutrients and energy</td>
</tr>
</tbody>
</table>

Our results in Chapter 3 and Chapter 4 reiterate the findings from metaflammation studies that hepatic ER stress and inflammasome activation augment liver damage (Hotamisligil, 2010), suggesting that this is a pervasive pathology regardless of the types of insult.

Furthermore, in Chapter 4, we demonstrated that metaflammation can be the pathological basis of augmented cell damage and organ dysfunction after severe trauma injury. While ER stress and UPR, as well as inflammasome activation and pro-inflammatory responses after the injury are protective and contributing to the clearance of pathogens and restoration of the homeostasis in healthy individuals, such pathological changes in addition with the existing metaflammation in obese mice are always detrimental, leading to significant impairment of the bioenergetics and aggravation of cell and organ damage.

Moreover, considering the self-limiting nature of the immunometabolic disorders after trauma, the comparative studies between this and that of metaflammation may provide some clue on converting persistent and refractory pathology of metaflammation to a curable one.
6.1.3 Inter-organ crosstalk between adipose tissue and liver: lipolysis and hepatic fat infiltration

In recent years, the uncoupling of oxidative phosphorylation, which is mediated by uncoupling proteins (UCPs), has become a hot topic of metabolism research, especially in the studies of adipose tissue pathology (Bouillaud et al., 2016). In contrary to most of the UCPs studies taking them as potential remedies against increased lipid deposition due to the excessive caloric intake in the treatment of metabolic diseases, uncoupling of oxidative phosphorylation is currently regarded as a mechanism of leakage of energy reserve under stressful conditions after severe trauma and thus is detrimental to trauma patients (Abdullahi and Jeschke, 2017; Patsouris et al., 2015; Sidossis et al., 2015). While the study of such browning of the adipose tissue after trauma is out of the scope of the current program, I would propose that this is not the only adipose tissue pathology (if it finally proves to be a ‘pathology’) contributing to metabolic disorders after trauma.

I have been particularly interested in the increased lipolysis of white adipose tissue (WAT) and its contribution to the hepatic fat infiltration (Chapter 2 and Chapter 4). We demonstrated increased lipolysis of WAT after major burn injury, which is correlated with the increased ER stress, inflammasome activation, and apoptosis in the adipose tissue. Mechanistically, we found that increased lipolysis in WAT is attributed to the suppression of inhibitory phosphorylation of HSL at Ser565 as the result of suppression of its upstream regulator AMPK, rather than the direct activation of the lipolysis-related phosphorylation of either HSL at Ser563 and Ser660 or MAPK at Thr202/Tyr204 and Thr185/Tyr187. Our finding
was later supported by the other studies on cancer cachexia which seems to share the same mechanism of the activation of lipolysis (Rohm et al., 2016).

Indeed, there is an increasing appreciation of the aberrant lipid signaling and lipotoxicity in the ectopic lipid accumulation such as in the liver and muscle tissues and their significance in the pathogenesis of metaflammation (Ertunc and Hotamisligil, 2016; Fu et al., 2012). By the same token, it cannot be emphasized enough the significance of stress activated increase of the lipolysis in WAT and its contribution to the hepatic fat infiltration, ER stress, immunological and metabolic derangement, and cell damage after major burn injury.

Moreover, we should always keep in mind that the macrophages are the major prosecutors of the inflammasome activation. Although my PhD research program mainly focuses on hepatocyte pathology, we do have a side project to test the macrophages polarization upon the palmitate treatment (Xiu et al., 2016). Our in vitro study demonstrated that the response of macrophages toward palmitate treatment depends on the differentiate status of the cells and, at least, differentiated macrophages (this may include the Kupffer cells, the resident macrophages in the liver) are pro-inflammatory and may contribute to the hepatic inflammasome activation under stress conditions.
6.1.4 “Birth and death, concomitant processes”*

Perhaps the most significant contribution of the current PhD research to the science community is our discovery of the connection between the liver regeneration under stress condition and aberrant immunological and metabolic signaling after severe trauma (Chapter 5).

Based on recent advancement of biotechnology, we are able to lineage-trace the hepatic progenitor cells (Kopp et al., 2011) which are proved to proliferate under the conditions of profound hepatic stress and liver damage and are able to restore functional liver parenchyma (Font-Burgada et al., 2015). In the context of pathological changes after major burn injury, our current study connects the aberrant liver regeneration by such an increased proliferation of facultative hepatic progenitor cells with their up-regulation of the pro-inflammatory signaling and stressful metabolic signaling. In doing so, we elucidate an important mechanism underlying the persistent yet recoverable hypermetabolism in major burned patients.

In the time course study of the liver regeneration after 30% TBSA thermal injury, we demonstrated 1) the increased proliferation and differentiation of the periportal ductal progenitor cells (PDPCs) peaking around two weeks post-burn; 2) the correlation of cellular stress response with the proliferation and differentiation of the PDPCs; 3) the contribution of the proliferation of the PDPCs to the hepatomegaly after the thermal injury.

It is commonly accepted that the liver is a regenerable organ and the hepatostat is always maintained for a fixed liver-to-body-weight ratio (Michalopoulos, 2017). However, it has
long been controversial how the liver regenerates (Carpentier et al., 2011; Font-Burgada et al., 2015; Michalopoulos et al., 2005; Planas-Paz et al., 2016; Wang et al., 2015). It is even more perplexed if considering the difference among the liver regeneration under normal or different pathological conditions such as after partial hepatectomy, or upon chronic liver injuries. Our mouse experimental study demonstrated that the hepatostat is perturbed after major burn injury with the significant increase of the liver-to-body-weight ratio and, by tracing the inducible Sox9-cre/ERT2: EYFP^+ cells, we observed proliferation and differentiation of the PDPCs contributing to the increase of the liver mass.

Our observations implicate not only that the severe burn injury is an insult strong enough to induce cellular stress response and cell damage and, as a result, stimulate the liver regeneration through PDPCs; but also that perturbation of the general homeostasis and increased metabolic demand post-burn may indirectly stimulate the liver regeneration to an extent that significantly beyond the limit of the hepatostat.

The home-run experimental study of the proliferation of the PDPCs post-burn is the microarray analysis of the comparison of the signaling pathways among PDPCs in sham, PDPCs in PBD7 mice and mature hepatocytes from PBD7 mice. We have shown that the LXR signaling pathway is repressed in PDPCs and their progeny after burn injury (PBD7) as compared with not only the PDPCs in sham but also the mature hepatocytes of the same time point of the 7th days post-burn. The significance of the repression of the LXR signaling pathway after major burn injury is two folds:
First, LXR is the master regulator of the reverse cholesterol transport pathway and key regulator of lipid homeostasis (Zhao and Dahlman-Wright, 2010). Down-regulation of LXR signaling pathway implicates impaired lipid homeostasis and aggravated hepatic fat infiltration. Interestingly, considering that LXRαβ-/- mice exhibits higher energy expenditure (EE) as well as higher UCP1 expression in brown adipose tissue (BAT) compared with WT mice in chronic settings (Korach-Andre et al., 2011), repression of LXR signaling pathway may directly or indirectly contribute to the browning of WAT and, in turn, hypermetabolism post-burn.

Second, down-regulation of the LXR signaling pathway is associated with the activation of genes linked to pro-inflammatory responses and this is usually referred to as trans-repression (Kidani and Bensinger, 2012). This is consistent with the result of our microarray analysis of the activation of the pro-inflammatory responses pathways and acute phase response pathways which are all contributing to increased catabolism post-burn.

We also confirmed these finding by Western blotting and densitometric analysis of the key modulators and effectors of the LXR and pro-inflammatory signaling pathways.

Overall, the increased proliferation of the PDPCs and the repression of the LXR signaling pathways in these cells mechanistically unify our two hypotheses on hepatomegaly post-burn: both increased proliferation of the PDPCs and hepatic fat infiltration present. More importantly, this sheds light on the effective intervention to ameliorate overwhelming pro-inflammatory responses and persistent hypermetabolism after major burn injury by administration of LXR agonists.
To a broader sense, our study demonstrates a paradigm of how the acute cellular stress response can give rise to a long term effect of metabolic changes in highly regenerable tissues and organs where the stem cell physiology should be taken into concern. It is out of the scope of our study whether such a change could be accumulative and contribute to more general pathological conditions. Nevertheless, further investigations to understand the cellular and molecular mechanisms connecting cellular stress response and changes in cell metabolism and cell fate are of paramount significance since all living organisms exist in ever changing environment and stress response to such perturbation of the environment is inevitable.

*: quote from ancient Chinese Daoist philosopher, Zhuangzi (369-286, B.C.)
6.2 Conclusions

In summary, we can conclude below points from the current PhD research project:

1. Major burn injury leads to ER stress, inflammasome activation, and increased apoptosis in the white adipose tissue, contributing to increased lipolysis (Chapter 2).

2. Major burn injury leads to hepatic ER stress, NLRP3 inflammasome activation, metabolic dysfunction and liver damage (Chapter 3 and Chapter 4).

3. Increased lipolysis in the white adipose tissue contributes to hepatic fat infiltration and augments liver dysfunction and liver damage after major burn injury (Chapter 2, 3 and 4).

4. Metaflammation in morbid obesity can be the pathological basis and augment hepatic cell damage and metabolic impairment after major burn injury (Chapter 4).

5. Perturbation of homeostasis and cellular stress response correlate with increased proliferation and differentiation of periportal ductal progenitor cells in the liver which are active in pro-inflammatory signaling and metabolic stress signaling, contributing to the persistent pro-inflammatory response and hypermetabolism after major burn injury (Chapter 5).

6. It is strongly implicated that LXR signaling pathway could be a therapeutic target for the early intervention of immunological and metabolic disorders in severe trauma patients (Chapter 5).

Putting all the above points together, we conclude that hepatic stress response, liver damage and regeneration contribute to persistent pro-inflammatory response and hypermetabolism after major burn injury (Figure 6.1).
Figure 6.1  Hepatic stress response, liver damage and regeneration contribute to persistent pro-inflammatory response and hypermetabolism after major burn injury.

There is a common pathology of ER stress, inflammasome activation and apoptosis in both liver and white adipose tissue after severe burn injury, as the result of cellular stress response and inflammation. Increased lipolysis of white adipose tissue also contributes to hepatic fat infiltration; together with hepatic mitochondrial dysregulation, contributing to liver dysfunction and damage. Hepatic stress response and liver damage trigger periportal ductal progenitor cells proliferation, all contributing to hepatomegaly and persistent pro-inflammatory state and hypermetabolism after major burn injury.
6.3 Limitations of the current study and future directions

I would consider below two points as the limitations of the current study and much could be done accordingly in the future studies:

1. Rodent animal experiments. While we are taking the many advantages of the rodents as the animal models for the in vivo study including significant shorter time for breeding and maturation of the animals and easy availability of the genetic modified strains (Vandamme, 2014), precaution should always be taken to interpret the observation we get from the animal study for answering the clinical questions for the patients due to the significant difference of anatomy and physiology between the animals and human (Abdullahi et al., 2014). For instance, in the two-hit rat model of burn plus LPS, one dose of sub-lethal LPS intraperitoneal injection was applied to mimic the endotoxemia and septic response after the initial insult of burn injury and subsequent hypovolemic shock. However, such a one dose LPS treatment could neither reflect the real scenario of continuous endotoxemia due to burn wound infection, nor cover the real complexity of burn infection in which not only Gram- bacteria but also Gram+ bacteria and fungi are commonly presented. In the case of conducting clinical trials to test the efficacy of the LXR agonists on the amelioration of the pro-inflammatory responses and metabolic disorders post-burn, additional consolidated animal experiments should be conducted beforehand including examining the response of LXR knock out mice on burn injury as well as LXR agonist administration to the Sox9-cre/ERT2: EYFP+ mice after burn injury. Especially, when the time points of the observation of the animal experiments are chosen, it is very challenging to accurately define the equivalency of what we see in the animals to the human pathology. Multiple time points should thus be included in the future studies.
2. Microarray transcriptomics analysis. The results and conclusions we present here is only from a small part of the microarray data. We can look forward to more findings from further data mining and analysis. However, since microarray analysis can only determine the fixed number of pre-defined genes (34472 genes in the current study), the read-out of the analysis is not objective and inclusive. If RNA sequencing can be done, more information could be available including long non-coding RNAs, micro-RNAs, and RNA modifications such as splicing and cleavage. These are all very important for the mechanistic study of the gene transcriptional regulation and control. Also, more accurate information could be available since the copy numbers can be collected directly without the possible skew of the information via PCR amplification. Moreover, we can look forward to more comprehensive understanding of the dynamic changes of the signaling pathways by transcriptomics profiling if PBD14, 21, 28 and 42 samples can be included for the analysis.

Except for the above 2 points of future direction which are generated immediately from the limitation of the study, I would consider answering below questions in the future investigation:

1. How cellular stress triggers stem/progenitor cells’ proliferation? How the changes in mitochondrial biology in hepatocytes and progenitor cells contribute to, or connect to the liver regeneration? I am intrigued to investigate the mechanisms of how cellular stress triggers stem/progenitor cells proliferation. There are multiple hypotheses including the depletion of the autophagy capacity in stem cell upon cellular stress (Garcia-Prat et al., 2016; Madrigal-Matute and Cuervo, 2016), rigorous UPR in (hematopoietic)stem cells for the clearance of
damaged stem cells whereas promotion of the differentiation of the progenitors (van Galen et al., 2014), and metabolic reconfiguration between glycolysis, mitochondrial oxidative phosphorylation and oxidative stress during the maturation of the stem cells (Shyh-Chang et al., 2013), etc. It is important and will be interesting to conduct experimental studies to obtain a confirmative answer(s) to this question.

2. How neurological signals are transduced to activate pathophysiological changes in liver and adipose tissue? It has long been accepted that severe burn injury activates sympathetic system which contributes to pro-inflammatory responses and hypermetabolism (Kulp et al., 2010; Wilmore et al., 1974). The activation of sympathetic system is one of the earliest physiological responses to the severe injury and nonselective β-adrenergic receptor antagonist (propranolol) has been used for many years for the treatment of the major burn patients (Herndon et al., 2012). However, nonselective β antagonist has wide range of pharmaceutical effects and there are always some unwanted ones to the critically ill patients. More specific intervention in this line is warranted. Recent studies on the sympathetic neuron-associated macrophages indicated its activation in metaflammation contributes to the destruction of the norepinephrine signaling and increased adipose tissue mass in obesity (Pirzgalska et al., 2017). It is worthwhile to conduct research in line with this mechanistic study for the possible liver and/or adipose tissue specific interventions.

3. What is the relationship or cross-talk among the hepatocytes, PDPCs and Kupffer cells, stellate cells and other stromal cells in the process of liver regeneration under stress conditions? There is a growing interest of the cross-talk among different cell types in the liver for the
immunometabolic study (Kotas and Medzhitov, 2015; Nowarski et al., 2017). Indeed, as we investigate the mechanisms of the proliferation and differentiation of the PDPCs upon stress, inflammation and cell damage, it is always important to keep in mind the changes in Kupffer cells and stromal cells and their impact on the cell biology of the PDPCs, although studies to answer the question of intercellular cross-talks will be complex and difficult.
References


pathways are involved in lipopolysaccharide-induced lipolysis in human adipocytes. Innate immunity 18, 25-34.


Kidani, Y., and Bensinger, S.J. (2012). Liver X receptor and peroxisome proliferator-activated receptor as integrators of lipid homeostasis and immunity. Immunological reviews 249, 72-83.


