EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF *Plasmodium falciparum* HSP70-1

A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

UNIVERSITY OF ZULULAND

By

Ofentse Jacob Pooe

November

2014

ABSTRACT

Heat shock proteins (Hsps) are conserved molecules that constitute a major part of the cell's molecular chaperone system (protein folding machinery). *Plasmodium falciparum* Hsps play an important cytoprotective role ensuring that the malaria parasite survives under the harsh conditions that prevail in the host environment. *P. falciparum* Hsp70-1 (PfHsp70-1) is a ubiquitous, cytosol-localised Hsp70 that is essential for parasite survival. Apart from their role as molecular chaperones, it is believed that some Hsps of parasitic origin are capable of modulating host immunity through signal transduction (chaperokine role). Most investigations focusing on the chaperokine functions of Hsps use recombinant forms of the proteins produced in *E. coli*. The main drawback is that the recombinant proteins co-purify with lipopolysaccharides (LPS). Although LPS removal techniques have been developed, they do not completely remove these contaminants, leading to confounding data as LPS are active immune modulants. The current study sought to investigate the immunomodulatory role of PfHsp70-1. A recombinant form of the protein was produced in three bacterial expression hosts (*E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *Brevibacillus choshinensis*). The protein was expressed attached to an N-terminal polyhistidine tag to facilitate purification by nickel affinity chromatography. PfHsp70-1 produced using the *E. coli* ClearColi BL21 and *Brevibacillus* expression systems was associated with no detectable traces of LPS. The protein exhibited no immunomodulatory function when it was exposed to macrophage cells cultured *in vitro*. However, PfHsp70-1 expressed in *E. coli* XL1 Blue was tainted with LPS contaminants and exhibited apparent immunomodulatory function suggesting that the LPS background was responsible for the signal. Findings, from this study, suggest that endotoxin-free PfHsp70-1 does not possess immunomdulatory function. Furthermore, this study confirms that *E. coli* ClearColi BL21 and *Brevibacillus* expression are

reliable expression hosts for the production of recombinant protein for use in immunomodulatory studies. Furthermore, cytokine production was induced on PMN cells that were exposed to a protein preparation consisting of the N-terminal ATPase subdomain of PfHsp70-1. However, the ATPase subdomain is known to be aggregation prone. thus, this may explain its apparent immune modulatory function. Polymyxin-B has routinely been used to neutralise the adverse effects of LPS from recombinant Hsps produced using traditional *E. coli*. Polymyxin-B is a cationic cyclic antibiotic that binds and aggregates LPS. However, the effects of polymyxin-B treatment on the integrity of the chaperones during LPS removal is still unclear. This study, therefore, sought to investigate the effect of polymyxin-B on PfHsp70-1's chaperone role, by investigating its effect on the thermal stability and structural conformation of PfHsp70-1. Results from this study clearly indicate that polymyxin-B interacts and interferes with the chaperone function of PfHsp70-1. Thus polymixin-B could potentially inhibit PfHsp70-1 function, thus, interfering with parasite growth. In, Conclusion this report demonstrates that recombinant forms of PfHsp70-1 can easily be produced without LPS contamination using *E. coli* ClearColi BL21 and *Brevibacillus* expression systems. Furthermore this study demonstrates that PfHsp70-1 proteins do not have immunomodulatory activity and that previously reported activity could have been due to the presence of co-purified LPS. Lastly this report discusses the prospects of using polymyxin-B's as a potential antimalarial therapeutic drug.

DECLARATION

I, Ofentse Jacob Pooe, declare that this is my own unaided work hereby submitted for the degree of Doctor of Philosophy at the University of Zululand. It has not been submitted before for any degree or examination at any other university.

_____

Ofentse Jacob Pooe

This _____ day of _____2015

DEDICATION

This thesis is lovingly dedicated to

My Mother, Selina Nanka Pooe

ACKNOWLEDGEMENTS

I will to thank God for giving me the strength to complete this study. I am extremely grateful to my supervisor Prof. Addmore Shonhai for his guidance, expertise and mentorship all through the duration of the project. Your support and encouragement indispensably made it possible for me to finish this project.

I also wish to acknowledge the following people for the technical support and for hosting me during research visits: Dr. Jude Pryzyborski, Dr. Gabi Koellisch (Phillip University, Marburg, Germany), Prof. Dr. Tamas Laskay (Lubeck University, Lubeck, Germany), Prof. Dean Gold-ring (University of KwaZulu Natal, South Africa) and Prof. Dr. Holger Heine (Borstel Research Centre, Germany).

I wish to extend further acknowledgement to the:

National Research Fund of South Africa for the PhD Innovation bursary and the "equipment and mobility" travel grant.

University of Zululand Research Committee (South Africa) for funding.

I would also like to express my gratitude to Mr. Mathula Ngwenya (University of Zululand, South Africa), for going out of his way to provide technical support, Mr. Robert Krause (University of KwaZulu Natal, South Africa), Mr. Tawanda Zininga, Mr. Stanley Makumire, Dr. Adelle Burger (University of Venda, South Africa) and Prof. T. Djarova (University of Zululand) for proof-reading my thesis.

TABLE OF CONTENTS

Abstract i
Declaration iii
Dedication iv
Acknowledgements v
Table of Contents vi
List of Figures x
List of Tables xiii
List of Symbols xiii
List of Outputs xiv

CHAPTER 1: Literature review 1

1.1 Global malaria impact 2
1.2 Malaria parasite biology 3
1.2.1 Life cycle of *Plasmodium falciparum* 3
1.3 Host cell remodelling by malaria parasites 5
1.3.1 The parasitophorous vacuole and tubulovesicular network 5
1.3.2 The apicoplast 7
1.3.3 Maurer's clefts 7
1.4 Molecular chaperones 8
1.4.1 Heat shock proteins as molecular chaperones 9
1.4.2 Hsp40 family of proteins 10
1.4.3 Hsp70 family of proteins 11
1. 5 *P. falciparum* Hsp70-1 and its homologue proteins 15
1.5.1 *P. falciparum* Hsp70 proteins that localize to the parasite cytosol 15
1.5.2 *P. falciparum* Hsp70s localised to the endoplasmic reticulum 20
1.5.3 *P. falciparum* Hsp70 proteins restricted to the mitochondrion 21
1.5.4 *P. falciparum* Hsp70s exported to the erythrocytes cytosol 21
1.6 Malaria heat shock proteins as potential drug targets 23
1.7 Role of molecular chaperones in malaria immunology 25

1.8 Toll-like receptors are vital in immunomodulation 27
1.9 Hsp70 proteins inducing the immune system 29
1.10 Problem Statement and motivation 32
1.11 Research Hypothesis 32
1.12 Broad objectives 33

CHAPTER 2: Expression and purification of functional PfHsp70-1 protein in the absence of LPS contamination 34

2.1 Introduction 35
2.2 Experimental procedures 41
2.2.1 Materials 41
2.2.2 Confirmation of pQE30/PfHsp70-1 plasmid construct encoding PfHsp70-1 42
2.2.3 Construction of plasmid expressing PfHsp70-1_{NBD} 42
2.2.4 Confirmation of pKK223-3/PfLDH encoding PfLDH protein 43
2.2.5 Cloning strategy of pNI-His/PfHsp70-1 towards expression and purification 43
2.2.6 Expression of recombinant proteins using *E. coli* XL1 Blue and *E. coli* ClearColi™ BL21 DE competent cells 43
2.2.7 Expression of recombinant PfHsp70-1 *B. choshinensis* 44
2.2.8 Purification of PfHsp70-1, PfHsp70-1_{NBD} and PfLDH recombinant proteins 45
2.2.9 Removal of LPS from PfHsp70-1 from *E. coli* XL1 Blue 46
2.2.10 The effect of ATP on LPS content during recombinant protein purification assay 46
2.2.11 Biological activity assay to determine the functional status of PfHsp70-1 purified without LPS contamination 47
2.2.12 Statistical Analysis 47
2.3 Results 49
2.3.1 Confirmation of pQE30/PfHsp70-1 plasmid encoding PfHsp70-1 49
2.3.2 Confirmation of pQE30/PfHsp70-1_{NBD} plasmid 49
2.3.3 Confirmation of pNI-His/PfHsp70 encoding PfHsp70-1 50
2.3.4 Confirmation of pKK223-2/PfLDH plasmid encoding PfLDH 51
2.3.5 Expression of PfHsp70-1 in Brevibacillus choshinensis 52
2.3.6 Purification of PfHsp70-1 expressed in *E. coli* XL1 Blue, *E. coli* ClearColi

BL21 and *B. choshinensis* 53
2.3.7 Expression and purification of PfHsp70-1_{NBD} 55
2.3.8 Expression and purification of PfLDH 56
2.3.9 Addition of ATP reduces LPS co-purification on PfHsp70-1 56
2.3.10 PfHsp70-1 purified in the absence of LPS contamination using *E. coli* ClearColi BL21 and *B. choshinensis* cells 58
2.3.11 PfHsp70-1 produced in the absence LPS are biologically active 59
2.4 Discussion 61

CHAPTER 3: Exploring the immunomodulatory function of recombinant *Plasmodium falciparum* Hsp70-1 64
3.1. Introduction 65
3.2. Experimental procedures 68
3.2.1 Materials 68
3.2.2 Cell cultures and stimulation assays 68
3.2.3 Cytokine expression analysis 70
3.2.4 Flow cytometry analysis 71
3.3. Results 72
3.3.1 Assessment of immune cell activation role 72
3.3.2 Effect of polymyxin-B on the cytokine stimulation activity of LPS contaminated recombinant PfHsp70-1 74
3.3.3 Protein structural integrity is required for PMN activation 76
3.4. Discussion 79

CHAPTER 4: Investigating the effect of polymyxin-b on PfHsp70-1 chaperone role 82
4.1 Introduction 83
4.2 Experimental procedures 86
4.2.1 Materials 86
4.2.2 Investigating the effect of polymyxin-B on the thermal stability of PfHsp70-1 86
4.2.3 Investigating the effect of polymyxin-B on the structure of PfHsp70-1 using limited proteolysis 87
4.2.4 Investigating the effect of polymyxin-B on PfHsp70-1 using native-PAGE 87

4.3 Results 89
4.3.1 Polymyxin-B promotes PfHsp70-1 aggregation 89
4.3.2 Polymyxin-B interferes with the chaperone function of PfHsp70-1 *in vitro* 90
4.3.3 PfHsp70-1's susceptibility to proteolysis is enhanced in the presence of polymyxin-B 91
4.3.4 Polymyxin-B's interaction with PfHsp70-1 induces a mobility shift on Native-PAGE analysis 92
4.4 Discussion 94

CHAPTER 5: Conclusion and future prospects 97
5.1 Conclusion and future work 98
5.2 Limitations to the experimental approach and how they could be overcome for future projects 99

REFERENCES 101

APPENDIX A: General experimental procedures 122
A. 1 Preparation of competent *E.coli* XL1Blue cells 123
A. 2 Transformation of competent cells 123
A. 3 Extraction of plasmid DNA 123
A. 4 Restriction digest of Plasmid DNA using enzymes 123
A. 5 Agarose gel electrophoresis 124
A. 6 Extraction of DNA from agarose gel 124
A. 7 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) 124
A. 8 Native polyacrylamide gel electrophoresis (native-PAGE) 125
A. 9 Western Blot 126
A. 10 Determination of protein concentration using Bradford assay 127
A. 11 Isolation of human peripheral blood granulocytes (PMNs) 127

APPENDIX B: **Supplementary Data 128**
B. 1 Bradford standard curve 128
B. 2 Phosphate standard curve 128

B. 3 Evaluating the effect of boiling on LPS 129

B. 4 Recombinant proteins produced in *E. coli* XL1Blue cells induce IL6 and IL12 production on DCs 130

B. 5 Recombinant proteins produced in *E. coli* ClearColi BL21 cells do not induce cytokine expression when on DCs 131

B.6 Cytokine induction analysis on TLR2 and TLR4 HEK293 cells treated with PfHsp70-1 produced in *E. coli* ClearColi BL21 and *Brevibacillus choshinensis* 132

B. 7 PfHsp70-1NBD produced in E. coli XL1Blue cells 133

APPENDIX C: List of reagents 134

LIST OF FIGURES

Figure 1.1 Countries with areas where malaria transmission occurs 2

Figure 1.2 *P. falciparum* life cycle 4

Figure 1.3 *P. falciparum* infected erythrocyte 6

Figure 1.4. Hsp40 domain organization and structure domain 11

Figure 1.5 Hsp70 domain organization and structure 12

Figure 1.6 Hsp70-mediated peptide refolding 14

Figure 1.7 *P.falciparum* Hsps70 protein localization within an infected erythrocyte 17

Figure 1.8 Anticipated protein domains for PfHsp70 homologues 18

Figure 2.1 Structural comparison of normal LPS with genetically modified ClearColi LPS 49

Figure 2.2 Restriction analysis of pQE30/PfHsp70-1 DNA plasmid 49

Figure 2.3 Restriction digest of pQE30/PfHsp70-1_{NBD} construct 50

Figure 2.4 Restriction digest of pNI-His/PfHsp70-1 construct 51

Figure 2.5 Restriction digest of pKK223-3/PfLDH construct 51

Figure 2.6 Intracellular and extracellular expression of recombinant PfHsp70-1 52

Figure 2.7 Expression and purification of recombinant PfHsp70-1 in the absence and presence of LPS 54

Figure 2.8 Successful expression and purification of PfHsp70-1_{NBD} 55

Figure 2.9 Expression and purification of recombinant PfLDH 56

Figure 2.10 The addition of ATP to PfHsp70-1 during purification reduces endotoxin contamination 57

Figure 2.11 Analysis of PfHsp70-1 endotoxin contamination 58

Figure 2.12 Analysis of the ATPase activity of PfHsp70-1 purified in the absence and presence LPS 60

Figure 3.1 PfHsp70-1 expressed from *E. coli* ClearColi BL21 do not induce cytokine release on macrophages cells 72

Figure 3.2 PfHsp70-1 expressed from *B. choshinensis* do not induce cytokine release on macrophages cells 73

Figure 3.3 Assesment of the effect of PfHsp70-1 on cytokine release by murine macrophages 74

Figure 3.4. Polymyxin-B suppresses cytokine stimulation activity of LPS contaminating recombinant PfHsp70-1 75

Figure 3.5. PfHsp70-1_{NBD} promotes L-selectin shedding and cytokine expression 79

Figure 4.1 Polymyxin-B structural representation 86

Figure 4.2 Polymyxin-B promotes PfHsp70-1 destabilization 92

Figure 4.3 Polymyxin-B induces the aggregation of PfHsp70-1 94

Figure 4.4 Partial proteolysis analysis of recombinant PfHsp70-1 digested in the presence and absence of polymyxin-B 95

Figure 4.5 Influence of polymyxin-B on PfHsp70-1 native-PAGE migration 96

Figure B. 1 Bradford standard curve for protein concentration determination 128

Figure B. 2 Phosphate standard curve for ATP hydrolysis analysis 128

Figure B. 3 LPS stimulatory activity is not hindered by boiling 129

Figure B. 4 Protein purified from E. coli XL1 Blue stimulates IL6 and IL12 production 130

Figure B. 5 Cytokine production on murine DCs cells incubated with protein produced in *E. coli* 131

Figure B.6 Analysis of IL8 production on TLR2 and TLR4 HEK293 cells treated with PfHsp70-1 produced in *E. coli* and *B. choshinensis* 132

Figure B.7 Successful expression and purification of PfHsp70-1_{NBD} in *E. coli* XL1Blue cells 133

LIST OF TABLES

Table 1.1 Classification of *P. falciparum* Hsp70 proteins 16

Table 2.1 Description of strains and plasmids used in this study 41

Table A.1 SDS-PAGE preparation 124

Table A. Preparation of native-PAGE 125

Table D.1 List of materials and specialised reagents 134

LIST OF SYMBOLS

Abbreviations of units	**Symbol Interpretation**
%	percent
µl	microlitre
A360	absorbance at 360 nanometres
A600	absorbance at 600 nanometres
bp	base pair
kDa	kilodalton
µM	micromolar
°C	degree Celsius
ml	millilitre
l	litres
w/v	weight per volume
v/v	volume per volume
µg	microgram
g	gram
α	alpha

LIST OF OUTPUTS

- **Publications in preparation**

Pooe OJ, Köllisch G, Heine H, and Shonhai A. Exploring the immunomodulatory function of *Plasmodium falciparum* Hsp70 expressed in various LPS minus bacterial strains bacterial strains.

Zininga T, **Pooe OJ** and Shonhai A. *Plasmodium falciparum* Hsp110 and Hsp70-1 interact in a nucleotide dependent fashion (submitted for review).

Pooe OJ, Köllisch G, and Shonhai A. Evaluating the aggregation suppression activity of polymyxin-B on PfHsp70-1.

- **Participations in Conferences**

Pooe OJ, Köllisch G, Heine H, and Shonhai A. Investigating of the immunomodulatory function of *Plasmodium falciparum* Hsp70 from various LPS minus bacterial strains. 28th Annual Symposium on the Protein Society, San Diego, USA, 27-30 July 2014.

Pooe OJ and Shonhai A. Investigating of the immunomodulatory function of *Plasmodium falciparum* Hsp70-1. The 8th Annual Science symposium, University of Zululand, South Africa, 1 November 2013.

Pooe OJ and Shonhai A. Evaluation of the immunomodulatory function of *Plasmodium falciparum* Hsp70. 6th MIM PAN-African malaria conference, Durban, South Africa, 6-11 October 2013.

CHAPTER 1

LITERATURE REVIEW

1.1 Global malaria impact

To date malaria is still regarded as a major public health problem across the world. The disease has undoubtedly had a great impact on world history than all other infectious diseases, particularly as it is a re-emerging disease (Morens and Fauci, 2013). According to the World health organization (WHO, 2010) report, malaria continues to be endemic throughout developing countries. Even though malaria has been eliminated in certain parts of the world, it is still a major problem across most tropical regions (Figure 1.1).

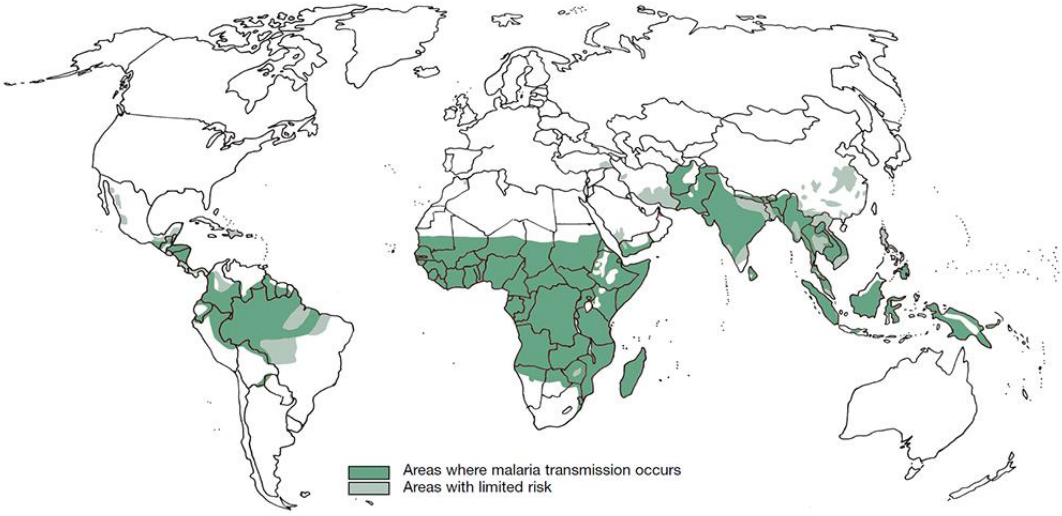


Figure 1.1 Countries with areas where malaria transmission occurs (WHO, 2010)

Malaria remains the world's most devastating disease for most people living in tropical areas. According to the WHO, in 2012 there were approximately 207 million malaria cases, leading to nearly 630 000 deaths across the globe. The disease is particularly fatal to pregnant women, as their infection leads to anaemia giving rise to low birth weight of their newborns (Schantz-Dunn and Nour, 2009). Malaria is an infectious disease characterised by recurring cycles of chills, fever, and sweating; with typical symptoms lasting 6–8 hours, for approximately three days. Malaria does not only affect individuals in malaria burdened countries; it further cripples their

financial state. According to the "Roll back malaria" report (WHO, 2012), a single malaria case costs a sum equivalent to ten working days in Africa. The financial loss in the form of malaria health care is estimated to be 1.3% of GDP growth per year in tropical Africa (WHO, 2010).

1.2 Malaria parasite biology

Malaria is caused by protozoa of the genus *Plasmodium*. The *Plasmodium* species exhibit a heterogeneous life cycle involving a stint in the vertebrate host and a phase in an arthropod vector (Daily *et al*., 2007). *Anopheles funestus* and *An.. gambiae,* are the most prominent malaria parasite vectors (Daily *et al*., 2007). Five parasite species of the *Plasmodium* genus are known to cause malaria in humans, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. falciparum* (White, 2008; Hector, 2014). Of these, *P. falciparum* is responsible for 90% of all malaria infections and malaria mortalities across the globe (Mendis *et al.*, 2001; Hector, 2014). Malaria parasites are transmitted from one host to another mostly through an insect vector (mosquito). Upon infecting the human host, sporozoites invade the hepatocyte cells inside the liver, where they develop into merozoites. These merozoites ultimately rupture from the hepatocytes and enter the bloodstream where they invade erythrocytes and begin a continuous asexual cycle (Figure 1.2).

1.2.1 Life cycle of *Plasmodium falciparum*

P. falciparum's life cycle (Figure 1.2) is extremely complex, in both the invertebrate (mosquito) vector and a vertebrate (human) host. During a blood meal, a mosquito injects the parasite sporozoites into the host's bloodstream (Figure 1.2; Fujioka and Aikawa, 2002). The inserted sporozoites travel through the peripheral blood system or lymphatic channels to infect the liver (Figure 1.2). After invading the liver, the sporozoites undergo asexual multiplication, to form

large intracellular cells known as schizonts (Figure 1.2; Scherf *et al*., 2008). After the schizonts mature, they rupture and release thousands of merozoites into the bloodstream. These merozoites subsequently infect the red blood cells; where they undergo their asexual life cycle inside the erythrocyte (Figure 1.2; Fujioka and Aikawa, 2002). Inside the erythrocyte, the parasite undergoes various developmental stages such as the ring stage, trophozoite and finally the parasites form schizonts containing numerous merozoites (Figure 1.2).

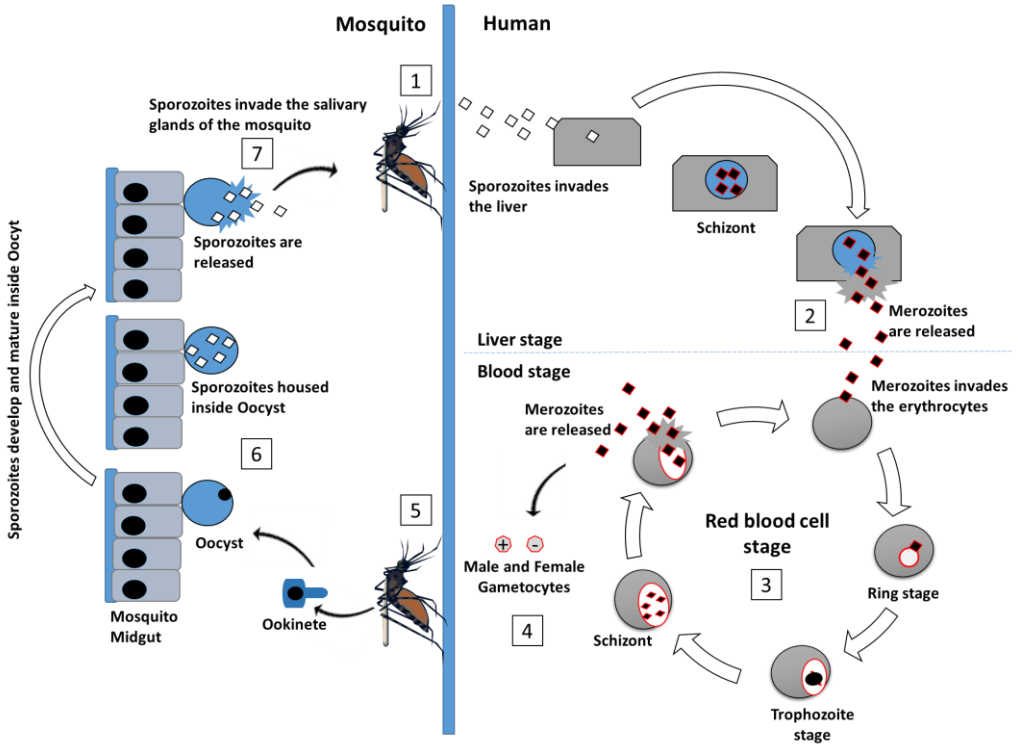


Figure 1.2 *P. falciparum* life cycle
An infected female *Anopheles* mosquito feeds on a human host, injecting parasites in the form of sporozoites into the host's bloodstream (1). The sporozoites invade liver cells, where each sporozoite undergoes asexual multiplication into thousands of merozoites (2). Merozoite-infected liver cells rupture, releasing merozoites into the bloodstream, where they invade erythrocytes and undergo their asexual life cycle. In the erythrocyte, the parasite transitions through various developmental stages such as the ring stage, trophozoite and finally schizont containing numerous merozoites. The merozoites rupture from the erythrocyte and are released into the bloodstream where they can invade previously uninfected erythrocytes (3). From the asexual stages in the blood stage, some parasites can differentiate into male and female gametocytes (4), which can be taken up by an uninfected mosquito (5). The zygote develops into an ookinete; that later develops into an oocyst containing sporozoites inside the midgut (6). After the oocysts mature, sporozoites are released into the mosquito salivary glands enabling the mosquito to infect a previously uninfected host (7). Adapted from Fujioka and Aikawa, 2002; Wirth, 2002; Scherf *et al*., 2008).

The merozoites rupture from the infected erythrocyte and some of them later re-infect erythrocytes (Figure 1.2; Wirth, 2002). Some of the parasite cells later differentiate into male and female gametocytes; these are ingested by uninfected mosquitoes during blood-meals or an infected host (Scherf *et al*., 2008). The zygote matures into an ookinete; that bores through the epithelium and develops into an oocyst containing sporozoites outside the mosquito midgut (Figure 1.2; Scherf *et al*., 2008). After the oocyst matures, sporozoites are released into the mosquito's salivary glands, enabling the mosquito to infect a previously uninfected host (Figure 1.2; Wirth, 2002). Following the infection of the cells, the parasite remodels the erythrocytes by exporting its proteins into the cell to form adhesive knobs on the surface of the infected erythrocytes (Bhattacharjee *et al*., 2012). These adhesive knobs allow the parasite to adhere to the blood vessels and evade the immune system (Bhattacharjee *et al*., 2012). This adhesive state of the parasite-infected erythrocytes is believed to be the primary factor that leads to rosetting associated with problems of malaria. Endothelial blood vessels may be blocked by the masses of these invaded erythrocytes. Adams and Turner (2002) proposed that the blockage of vessels by *P. falciparum*-infected erythrocytes leads to the development of cerebral malaria.

1.3 Host cell remodelling by malaria parasites

1.3.1 The parasitophorous vacuole membrane and tubulovesicular network

Malaria parasites manage to evade the immune system, by penetrating and replicating inside the host erythrocytes and the liver. *P. falciparum*-encoded produces proteins are essential for the remodelling of the infected erythrocyte (Mbengue *et al*., 2012; Lindner *et al*., 2013). These modifications enable the infected cell to evade splenic clearance by adhering to the blood vessel walls thorough adhesive knobs (Figure 1.3; Chen *et al*., 2000). After infecting the erythrocytes,

the *P. falciparum* parasite forms the parasitophorous vacuole membrane (PVM) to shelter the parasite while inside the erythrocyte (Figure 1.3; Lingelbach and Joiner, 1998; Eksi and Williamson, 2011). The PVM comprises of lipids derived from the plasma membrane of the host cell. The PV is a semi-permeable barrier between the parasite and the erythrocyte, facilitating nutrient acquisition and ion exchange. lPV also acts as a transportation compartment for parasite proteins that are exported into the infected erythrocyte cytosol (Figure 1.3; Nyalwidhe and Lingelbach, 2005; Charpian and Przyborski, 2008). Mackellar and co-workers (2010) proposed that *P. falciparum* PF10_0164 (ETRAMP10.3) may be a vital PVM protein, which could potentially be targeted for inhibition by antimalarial agents (Mackellar *et al*., 2010).

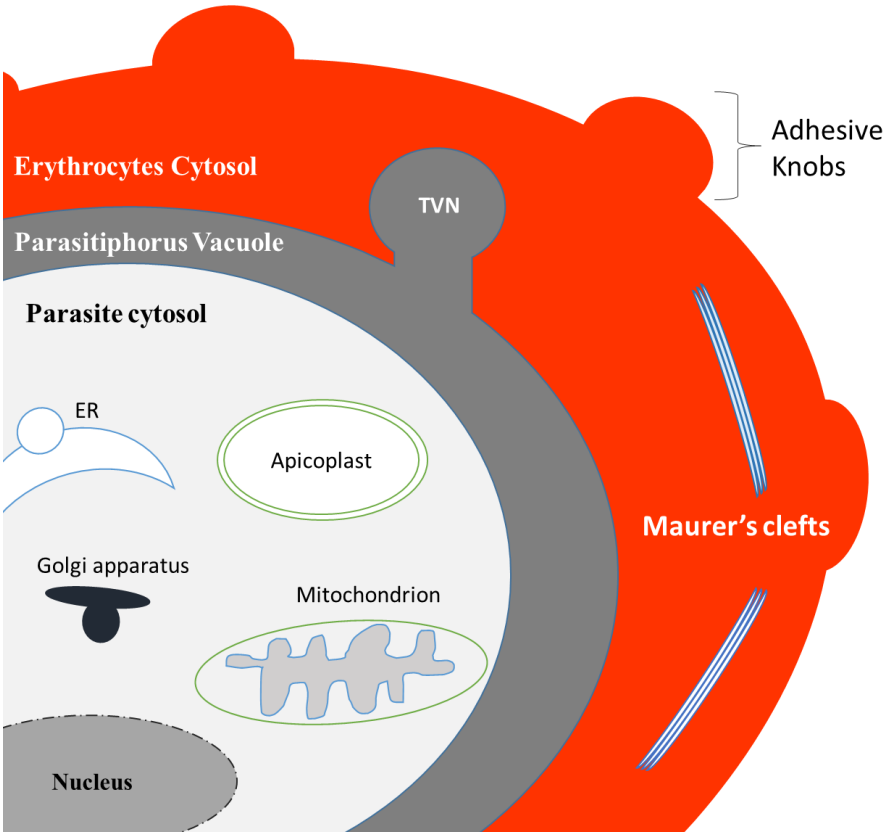


Figure 1.3 *P. falciparum* infected erythrocyte
A human erythrocyte infected by *P. falciparum.* Upon being invaded by the sporozoites or merozoites, the parasite forms the parasitiphorus vacuole within which the parasite resides and proliferates. The letters represent the following organelles; ER (endoplamic reticulum) and TVN (tubulovesicular network; adapted from Shonhai *et al.*, 2007.

The PVM extends to form tubular like membranous structures known as the tubulovesicular network (TVN). TVN's are believed to be involved in the recruitment of lipid and proteins needed for the development of the PVM (Figure 1.3). Interfering with the development of the TVN has been associated with blockage of the transport of nutrients to the parasite (Lauer *et al*., 1997; Tamez *et al*., 2008). Tamez and colleagues (2008) demonstrated that inhibiting the growth of the TVN does not kill the parasite but rather impairs intracellular parasite growth.

1.3.2 The apicoplast

Also located within the parasite is the apicoplast that is a plastid-like organelle which is absent in humans. Hence, it has been described as a potential drug target (Figure 1.3; Janouškovec *et al*., 2010). The apicoplast genome encodes house-keeping genes, such as tRNAs, ribosome subunits, RNA polymerase and proteins involved in protein translocation (Wilson *et al*., 1996; Yeh and DeRisi, 2011). The apicoplast is essential for both intra-erythrocytic and intra-hepatic *P. falciparum* growth in the human host (Dahl *et al*. 2006; Stanway *et al*., 2009; Yeh and DeRisi, 2011).

1.3.3 Maurer's clefts

In addition to the TVN, the Maurer's clefts are parasite-derived structures inside the infected erythrocyte cytosol. Goerg Maurer was the first to describe these structures in 1902, using a light microscope (Maurer *et al*., 1902; Mundwiler-Pachlatko and Beck, 2013). Maurer's clefts are believed to originate from the PVM but form a separate independent structure in the cytosol of the infected erythrocyte (Figure 1.3; Cyrklaff *et al*., 2011). Maurer's clefts are thought to play a role in protein sorting and transportation (Figure 1.3; Bhattacharjee *et al*., 2008; Sam-Yellowe,

2009). Some *P. falciparum*-encoded proteins that are involved in protein remodelling have been associated with the Maurer's cleft during their exportation to the erythrocyte membrane (Knuepfer *et al*., 2005; Dixon *et al*., 2011). Thus, the Maurer's clefts are thought to play a major role in the virulence of *P. falciparum*. Furthermore, the Maurer's clefts have been implicated in the translocation of parasite protein that remodel the infected host cell (McMillan *et al*., 2013). Mbengue and colleagues (2014) proposed that the expression of parasite molecular chaperones (section 1.5) may be associated with the host cell remodelling of an infected erythrocyte.

1.4 Molecular chaperones

Molecular chaperones were first described as nuclear proteins that facilitated disassembly of nucleosomes during amphibian egg formation (Laskey *et al*., 1978). Ellis (1987) later extended the term to include proteins that facilitate folding and assembly reactions. Molecular chaperones are involved in protein folding, unfolding and remodelling (Bukau *et al*., 2006; Saibil *et al*., 2013). Molecular chaperones are ubiquitous molecules that are expressed to counter the effects of cell stress, which could include heat shock, ionising radiation, UV light, oxidation or chemical stress (Saibil *et al*., 2013). The importance of molecular chaperones is enhanced under stressful physiological or non-physiological conditions. The role of molecular chaperones could, therefore, be summarised as to prevent nascent proteins from aggregating or misfolding and to aid in the assembly of relatively stable multiprotein complexes or large proteins (Caplan, 2003). The largest molecular chaperone group that assists protein folding and promotes cell survival are known as heat shock proteins (Hsp) (Bukau *et al*., 2006).

1.4.1 Heat shock proteins as molecular chaperones

The expression of heat shock proteins (Hsps) due to heat-stress was first reported by Ritossa (1962) using *Drosophila melanogaster* cells. Hsps are ubiquitous proteins that are needed to ensure the appropriate folding and conformation of other proteins in the cell (Kampinga *et al*., 2009; Storey and Storey, 2011). All living organisms respond to heat shock, oxidative stress or other stressful situations by increasing the expression of Hsps (Storey and Storey, 2011; Verghese *et al*., 2012). Hsps are classified according to their average molecular weight, with major classes consisting of small Hsps (sHsp), Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100 (Kampinga *et al*., 2009). Some Hsps, such as Hsp70 proteins are constitutively expressed to maintain cellular homeostasis. Due to the significant role molecular chaperones play in maintaining proteome stability, it is, therefore, not surprising that intracellular Hsps are found in most subcellular compartments such as the nucleus, cytosol, mitochondria and the endoplasmic reticulum (ER) (Kampinga *et al*., 2009; Pechmann *et al*., 2013).

Small Hsps play a vital role in signal transduction, cell differentiation, morphological development, protein anti-aggregation and the inhibition of apoptosis inducing stimuli during stress (Gusev *et al*., 2002, Basha *et al*., 2011, Hilton *et al*., 2012). Their sizes range from 9-43 kDa and they are functionally ATP-independent proteins (Hilton *et al*., 2012; reviewed in Edkins and Boshoff, 2014). Some sHsp function as holdases, as they can interact with unfolded peptide substrates to prevent their permanent aggregation (reviewed in Edkins and Boshoff, 2014). Unlike other chaperones, these molecules do not actively refold substates but rather they capture and hold the partially misfolded substrates to prevent further aggregation (McHaourab *et al*., 2009, reviewed in Edkins and Boshoff., 2014). Some sHsp are crucial for the development of

thermo-tolerence in cells (Plesofsky-Vig *et al*., 1995). Furthermore, they have been implicated in the normal functioning of chaperone complexes that passively bind and stabilise compromised proteins with high affinity (Garrido *et al*., 2006, McHaourab *et al*., 2009).

The Hsp60, Hsp70, and Hsp90 protein families are ATP-dependent chaperones, are primarily responsible for the refolding of nascent or unfolded polypeptides (Storey and Storey, 2011). In some cases, Hsps work in concert with other molecules known as co-chaperones, in order inhibit the aggregation of denatured proteins and assist in the refolding of denatured proteins. Co-chaperones facilitate the specificity of chaperone protein folding by serving as substrate selectors or regulating the nucleotide-bound state of chaperones. Co-chaperones regulate of the activity their partner chaperones by directing the conformation of the chaperone (Caplan, 2003; Blatch, 2007).

1.4.2 Hsp40 family of proteins

Hsp40 proteins are classified into four types according to the presence of a J-domain, GF domain and a zinc- binding domain (Cheetham and Caplan, 1998; Botha *et al*., 2007). Hsp40 proteins are characterised by the presence of the J-domain (Figure 1.5) (Huang *et al*., 1998). The J-domain, of Hsp40s is conserved and its role is to interact and stimulate the ATPase activity of Hsp70 proteins (Garimella *et al*., 2006). The *E. coli* DnaJ, has a well-conserved signature feature made up of histidine–proline–aspartic acid (HPD) motif (Figure 1.5). Variations within the HPD motif are thought to affect the Hsp40's ability to stimulate ATPase activity of Hsp70 (Wittung-stafshede *et al*., 2003). Hsp40 proteins are known to serve co-chaperones to Hsp70 proteins (Figure 1.3) and are believed to function as chaperones in their own right (Lu and Cyr, 1998).

Some Hsps40 have been shown to hold and refold, misfolded protein substrates (Stirling *et al*., 2003; Lu and Cyr, 1998). Hsp40 proteins have been shown to possess dual chaperone/co-chaperone capabilities.

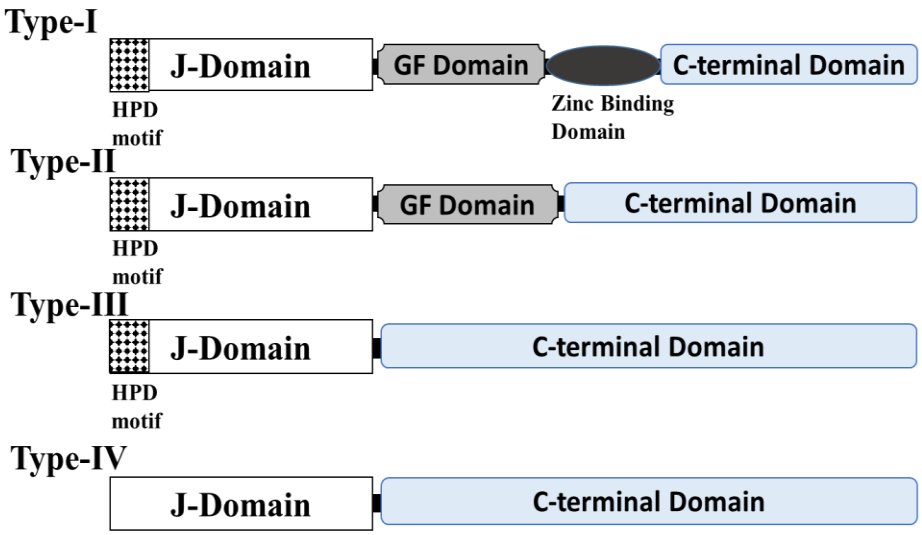


Figure 1.5 Hsp40 domain organization and structure
Hsp40 proteins are classified into four types according to the presence of a J-domain, GF domain and a zinc binding domain (Cheetham and Caplan, 1998; Botha *et al*., 2007). The HPD motif is typically present within the J-domain. Type I Hsp40, contains all four domains, type II lacks the zinc binding domain. Type III and type IV contain only the signature J-domain; however, type IV displays variations with the HPD domain.

1.4.3 Hsp70 family of proteins

Hsp70 proteins are molecular chaperones that are present in virtually all organisms and are by far the most studied of all Hsp families (Boorstein *et al*., 1994). These proteins are required for maintaining proteins homeostasis in the cell (Edkins and Boshoff, 2014). Hsp70 chaperones have also been shown to assist in protein translocation (Hamman *et al*., 1998) and assembly of protein complexes (Zylicz *et al*., 1989). These molecular chaperones also prevent protein aggregation or self-association by binding to hydrophobic segments of misfolded proteins (Mayer and Bukau, 2005; Kabani and Martineau, 2008). Hsp70 proteins consist of a 45 kDa N-terminal nucleotide

binding domain (NBD) and a 25 kDa peptide binding domain (PBD) (Figure 1.4; Wang *et al*., 1993). The NBD constitutes of four subdomains with two lobes namely the upper and the lower cleft (Figure 1.4 B; Flaherty *et al*., 1991; Bork *et al*., 1992). The Hsp70 NBD upper cleft houses the nucleotide binding site and is responsible for ATP hydrolysis resulting in the conformational changes of the Hsp70 (Rudiger *et al*., 1997). The NBD of Hsp70 regulates the conformation of the PBD, which interacts with a hydrophobic five residue segment of the substrate clients (Rudiger *et al*., 1997).

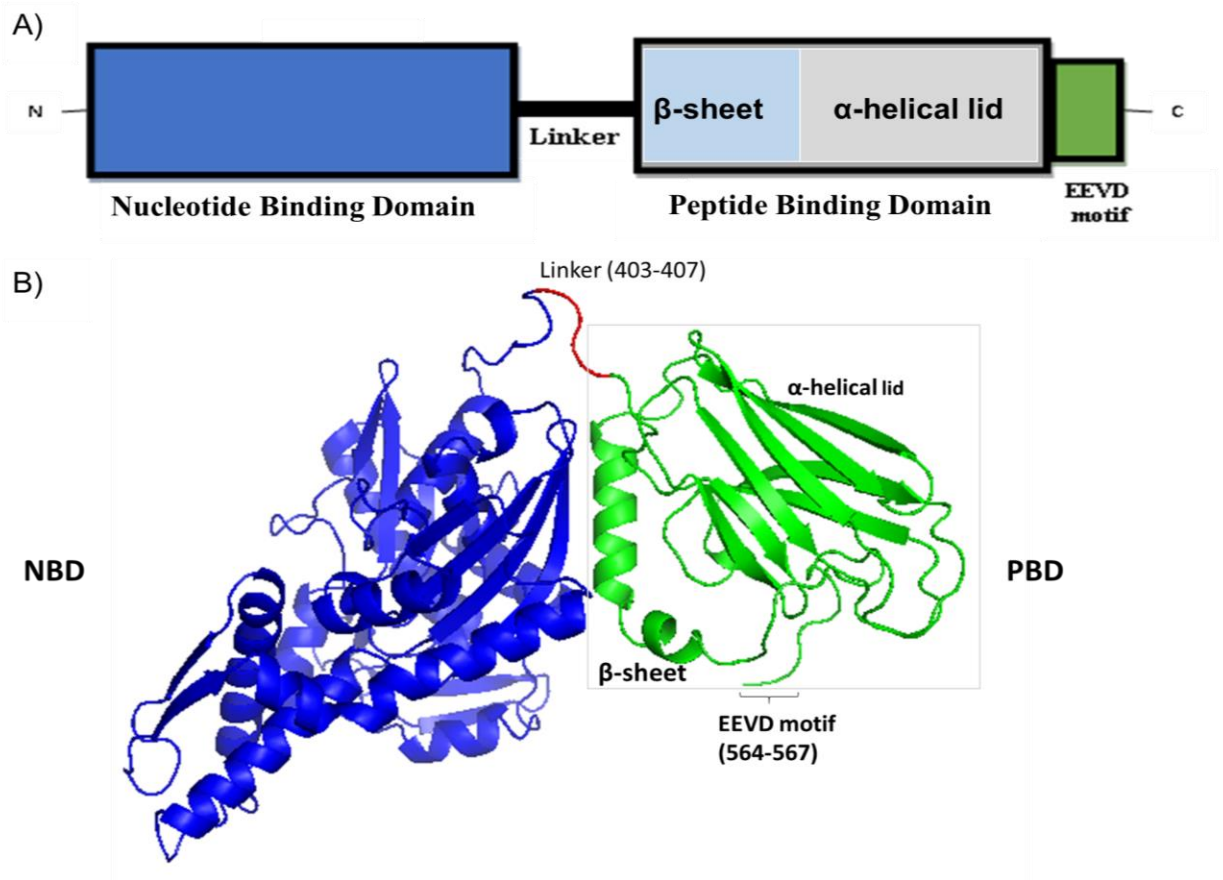


Figure 1.4 Hsp70 domain organization and structure
The figure shows the N-terminus NBD (45 kDa) attached to the PBD (25 kDa) connected by a highly charged linker domain (A). Located at the C-terminus of PBD is the EEVD motif, used to interact with substrate peptides (A). Structure of Hsc70 (PDB: 1yuw.1.A) (B). The PBD β-sheet binds and incorporates hydrophobic peptides and the α-helical lid locks the peptide into place in the hydrophobic pocket. Co-ordinate files of the structure were obtained from the Protein Data Bank (PDB; Bermann *et al*., 2003). The protein structure was then visualized using Pymol (Delano Scientific) (Sali *et al*., 1993; Arnold *et al*., 2006; Benkert *et al*., 2011; Marco *et al*., 2014).

These conformational changes regulate the substrate binding capabilities of the PBD (Buchberger *et al*., 1995; Fung *et al*., 1996). Jiang and colleagues (2005) proposed that the linker region adjoining the NBD and PBD is vital for communication between the domains (Figure 1.4 A). Furthermore, the mutation of residues in the linker region have been shown to interfere with the chaperone function of Hsp70 proteins (Han and Christen; 2001; Jiang *et al*., 2007). Functional differences between Hsp70 homologues are mainly due to the variable PBD and not the NDB, which is highly conserved (Wisniewska *et al*., 2010).The PBD is comprised of the hydrophobic peptide binding pocket constituted by a sandwich of β-sheets and a flexible α-helical lid subdomain (Figure 1.4 B; Zhu *et al*., 1996). The β-sheets forms the substrate binding subdomain and the α-helical subdomain, forms the lid structure that covers the PBD (Mayer and Bukau, 2005). The β-sheets recognises hydrophobic amino acids of misfolded proteins, as the exposure of these hydrophobic amino acids is often associated with a proteins' tendency to aggregate (Rousseau *et al*., 2006; Hartl *et al*., 2011). To refold a misfolded polypeptide, the Hsp40 co-chaperone binds and delivers these misfolded proteins to an ATP-bound Hsp70 (Figure 1.4; Kampinga and Craig, 2010). Hsp70 are known to operate in concert with Hsp100 in disaggregating large aggregated proteins.

Hsp40 (section 1.4.2) proteins are ubiquitously expressed and are found in various organisms; their primary role is to regulate ATP-dependant binding of substrates by Hsp70 (Cyr *et al*. 1992). Hsp40s are highly diverse proteins that specifically deliver unfolded substrates to Hsp70 proteins. When polypeptides are unfolded, it means that their generally concealed hydrophobic residues become exposed which could result in adverse interactions with other peptide or macromolecules or the complete aggregation of the unfolded polypeptide. To refold an unfolded

substrate, the co-chaperone Hsp40, binds and delivers these misfolded substrate proteins to an ATP-bound Hsp70 (Figure 1.6). The Hsp40-Hsp70 interaction is a low-affinity, transient one that stimulates the hydrolysis of ATP to ADP by Hsp70 (Figure 1.6; Szabo *et al*., 1994; Kampinga and Craig, 2010). In the ADP-Hsp70 state, Hsp70 has increased affinity for the substrate resulting in the peptide being refolded by Hsp70. The Hsp70-substrate association is stabilised by Hsp40, which stimulates the basal ATPase activity of Hsp70 through the NBD of Hsp70 (Jiang *et al*., 2007; Li *et al*., 2009). The Hsp70-ADP bound complex is then bound by a nucleotide exchange factor (NEF) which facilitates the allosteric Hsp70 cycle. This cycle allows switches between the Hsp70-ATP-bound and Hsp70-ADP-bound states through ATP hydrolysis and substrate exchanges. (Figure 1.6).

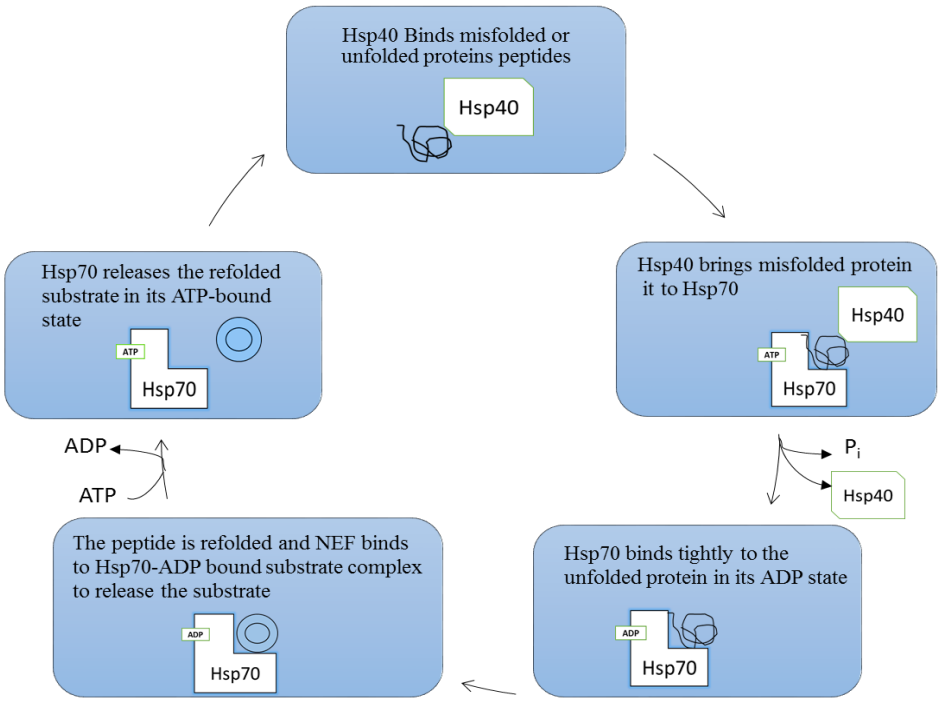


Figure 1.6 Hsp70-mediated peptide refolding
The unfolded/misfolded peptide is bound by Hsp40; that delivers it to the ATP-bound Hsp70. Hsp70 undergoes a conformational change, resulting in Hsp70 exhibiting an increased affinity for the misfolded substrate peptide being ADP-bound Hsp70. A nucleotide exchange factor (NEF) facilitates the exchange of ADP bound to Hsp70 using ATP. Which results in a conformational change with low substrate affinity, thus, releasing the refolded substrate (Hennessy *et al*., 2005).

This allosteric Hsp70 cycle allows Hsp70 to undergo a conformational change to release the refolded peptide substrate (Figure 1.6). Upon releasing the substrate, peptides that did not reach their proper folding state will rebind to Hsp70 until they are properly refolded, thus, preventing them from re-aggregation (Rousseau *et al*., 2006). Proteins that cannot reach their proper folded state after numerous cycles of binding to Hsp70 may be transferred to the specialised chaperonin cage-like structure for correct folding (Mayer and Bukau, 2005). Denatured proteins bound to Hsp70 that cannot be refolded are subjected to Carboxyl terminus of Hsp70-interacting protein (CHIP). CHIP, is an Hsp70 co-chaperone that targets denatured proteins to proteasome for proteolytic degradation (Kampinga *et al*., 2003).

1.5 Hsp70 proteins in *Plasmodium falciparum*

The *P. falciparum* genome encodes six Hp70 homologue proteins (reviewed in Njunge *et al*., 2013). *P. falciparum* Hsp 70-like proteins are localised in different sections of the parasite and carry out various functions (Figure 1.5; reviewed in Mbengue *et al*., 2014). *P. falciparum* Hsp70 has six homologues, PfHsp70-1 (PfHsp70_C, PF3D7_0818900), PfHsp70-2/PfBiP (PfHsp70_ER, PF3D7_0917900), PfHsp70-3 (PfHsp70_M, PF3D7_ 1134000), PfHsp70-x (PF3D7_0831700), PfHsp70-y (PF3D7_1344200) and PfHsp70-z (PF3D7_0708800) (Table 1.1).

1.5.1 Cytosol restricted *Plasmodium falciparum* Hsp70 proteins

P. falciparum Hsps 70-1 is a ubiquitous protein and essential in the parasite life-cycle and has been demonstrated to be important for the survival of *P. falciparum* (reviewed in Shonhai *et al*., 2011). PfHsp70-1 is also stress inducible protein that is known to be expressed through-out the parasite erythrocytic-stage (Patankar *et al*., 2001; Aurrecoechea *et al*., 2009). PfHsp70-1 is

predominantly located in the parasite cytosol. However, it has been proposed that it translocates to the nucleus during heat stress (Figure 1.7; Table 1.1; Kumar *et al*., 1991). Other scholars have suggested that PfHsp70-1 is also present within the PV and Maurer's clefts (Vincensini *et al*., 2005; Nyawidhe and Lingelbach, 2006). Recently, Grover and colleagues (2013) suggested that PfHsp70-1 was detectable in the cytosol of an infected erythrocyte. However, it is conceivable that the reported PfHsp70-1 protein outside the parasite cytosol may due to possible contamination from the subcellular fractions, as PfHsp70-1 is fairly ubiquitous protein. Similar to other cytosolic eukaryotic Hsp70 molecules, PfHsp70-1 contains a 45 KDa NBD and a 25 KDa PBD with an EEVD motif on the C-terminal (Figure 1.7).

Table 1.1 Classification of *P. falciparum* Hsp70 proteins

Names	M.W (kDa)	Localisation	Functions
PfHsp70-1	74	Parasite Cytosol[i] Nucleus[b]	-Protein folding[h j,k] -Essential protein[h]
PfHsp70-x	76	J-dots[l] Erythrocytes Cytosol[l]	-Exported to the RBC -Export and folding of *P. falciparum* proteins into the infected RBC (N.C)[l]
PfHsp70-z	100	Parasite Cytosol[n]	-NEF of PfHsp70-1 (N.C)[g] -Essential protein -Prevents aggregation of a *P. falciparum* Asn repeat containing protein[n]
PfHsp70-2/ PfBiP	73	Endoplasmic reticulum[b]	-Import of *P. falciparum* protein into ER (N.C)[g] -Protein folding and quality control in the ER (N.C)[g]
PfHsp70-3	73	Mitochondrion[d,e,g]	-Import of proteins into the mitochondrial matrix (N.C)[g,m]
PfHsp70-y	108	Endoplasmic reticulum[b]	-NEF for PfBiP (N.E)[g]

NC-not experimentally confirmed, RBC- red blood cell, NEF- nucleotide exchange factors, ER, Endoplasmic reticulum. The superscript letters represent the following references; a - Sharma, 1992; b - Kumar *et al*., 1991; c Nyalwidhe and Lingelbach, 2006; d -Sargeant *et al*., 2006, e -Slapeta and Keithly, 2004, f -Shonhai *et al*., 2005, g -Shonhai *et al*., 2007, h -Shonhai *et al*., 2008, i -Pesce *et al*., 2008, j -Bell *et al*., 2011, k -Stephens *et al*., 2011, l -Külzer *et al*., 2012 , m - Njunge *et al*., 2013 and n - Muralidharan *et al*., 2012 (adapted from Shonhai *et al*., 2007).

PfHsp70-1 is further distinguished from other PfHsp70 homologues by the presence of GGMP repeats on its PBD (Figure 1.8; Shonhai *et al*., 2007). The role of the GGMP repeats in PfHsp70-1 chaperone function is still unknown. However, parasitic GGMP motifs have been known to be highly immunogenic (Kumar and Zheng, 1998). In light of this, PfHsp70-1 may modulate the host immune system, should it leak into the bloodstream.

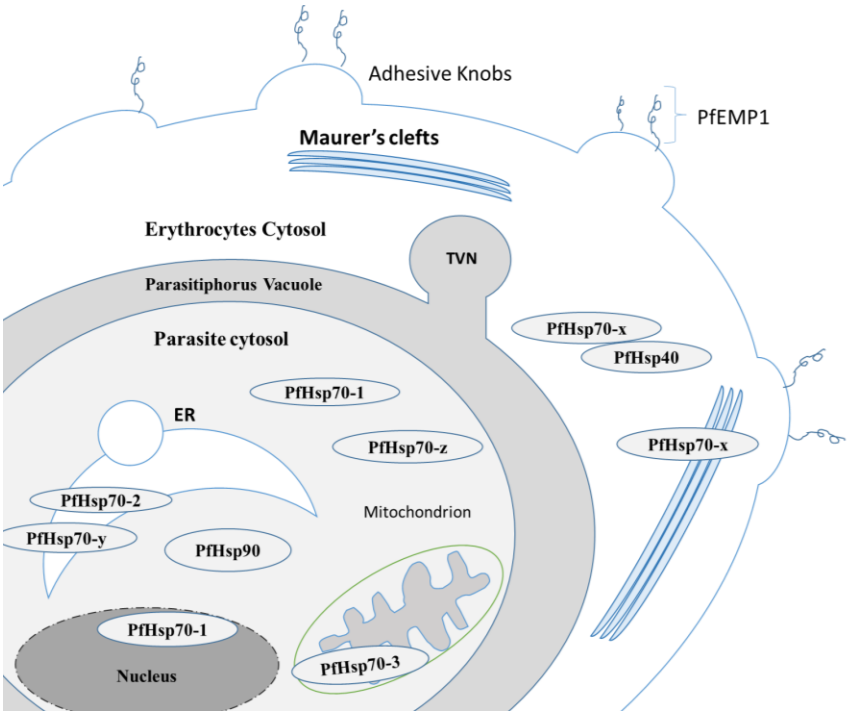


Figure 1.7 Parasite Hsps70 localisations within a falciparum-infected erythrocyte
Major Hsp locations within a *P. falciparum*-infected erythrocyte. After infecting the erythrocyte, some of the indicated parasite Hsps are exported into the erythrocyte cytosol in order to remodel the cell.

PfHsp70-1 has been shown to exhibit chaperone activity (Matambo *et al*., 2004; Shonhai *et al*., 2008). Shonhai and colleagues (2005) demonstrated the *in vivo* chaperone function of PfHsp70-1 by reversing the thermo-sensitivity of an *Escherichia coli* DnaK (prokaryotic Hsp70) mutant strain. Recombinant PfHsp70-1 was further used to inhibit the heat-induced aggregation of malate dehydrogenase (MDH) *in vitro* (Shonhai *et al.*, 2008). Misra and Ramachandran (2009) demonstrated the *in vitro* refoldase activity of PfHsp70-1, by actively refolding, previously

misfolded glucose-6-phosphate dehydrogenase and α-glucosidase. Its chaperone role was further demonstrated by co-expressing *P. falciparum* GTP cyclohydrolase I (PfGCHI) with PfHsp70-1.

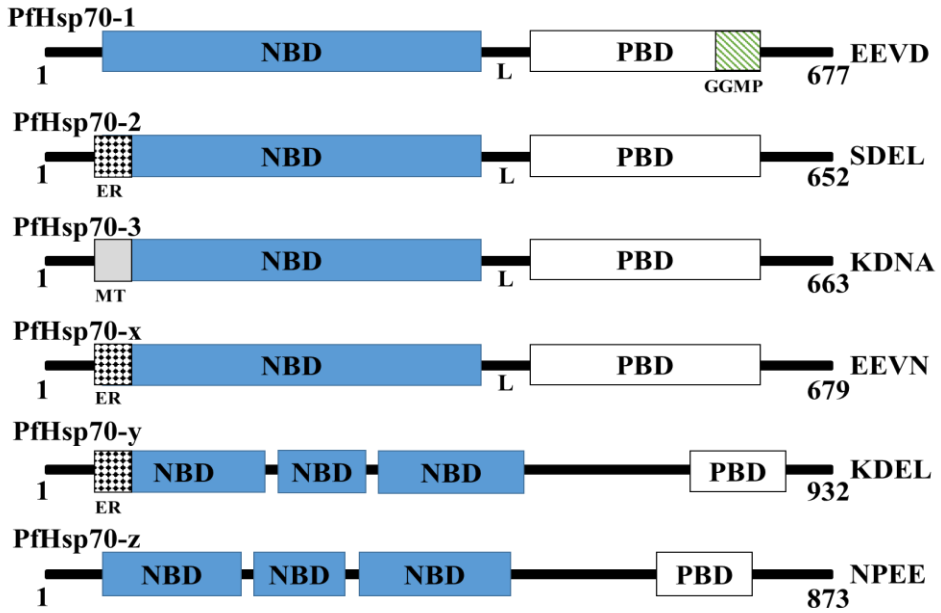


Figure 1.8 Anticipated protein domains for PfHsp70 homologues
Prediction of protein domains of six PfHsp70 homologue chaperones. PfHsp70-1 has an immunogenic GGMP repeat sequence. PfBiP, PfHsp70-x and PfHsp70-z have an ER signal peptide, whereas PfHsp70-3 has a mitochondrion targeting signal. PfHsp70-y and PfHsp70-z lack a distinct linker domain. The letter L represents the linker domain. ER and MT represent the endoplasmic reticulum and mitochondrion, respectively (adapted from Lui and Houry, 2014).

PfHsp70-1 was shown to promote the expression and solubility of recombinant PfGCHI in *E. coli* cells (Stephens *et al*., 2011). Co-expression with PfHsp70-1 improved the production and purification quality of plasmodial proteins (Stephens *et al*., 2011). PfHsp70-1 has also been shown to possess a functional linker domain (section 1.4.3), whose disruption resulted in the compromised function of the protein (Shonhai *et al*., 2005). PfHsp70-1 has been shown to have a significantly higher ATPase activity as compared to human-hsp70 (HSPA1A) (Matambo *et al*., 2004; Ramya *et al*., 2006). Misra and Ramachandran (2009) showed that PfHsp70-1 maintained optimal ATPase activity even at 50 °C. Hence, PfHsp70-1 is thought to aid *P. falciparum* survive fever episodes in the human body and is thus a potential drug target. PfHsp70-1 is produced at all

the erythrocytic stages of the parasite (Aurrecoechea *et al*., 2009). PfHsp70-1 has been shown to interact with nuclear-encoded proteins implicated in the replication of *P. falciparum* apicoplast DNA (Misra and Ramachandran, 2010). Furthermore, nuclear-encoded proteins destined for the apicoplast are equipped with PfHsp70-1 recognition motifs. Thus, PfHsp70-1 has been implicated in the trafficking of proteins to the apicoplast, ER and mitochondrion (Foth *et al*., 2003; Misra and Ramachandran, 2010; Njunge *et al*., 2013). However, Blatch and Pryzyborski, (2011) argued that since apicoplast proteins enter the secretory system at the ER, their transit peptides are potentially never exposed to cytosolic chaperones. The ER-resident Hsp70/BiP has been shown to interact with the transit peptide that translocates apicoplast targeted proteins (Yung *et al*. 2003). Hence, PfHsp70-1 may not be directly responsible for trafficking of proteins into the apicoplast. Heiny and colleagues (2012) further suggested that due to the high homology between the cytosolic PfHsp70-1 and ER-resident PfBiP, it is therefore likely that the reported interaction between PfHsp70-1 and apicoplast targeted proteins may have been an *in vitro* artefact (Heiny *et al*., 2012). Further investigations are still needed to elucidate how parasite proteins are transported into the apicoplast.

PfHsp70-z is also localised in the parasite cytosol (Figure 1.7) and is significantly larger than PfHsp70-1 (Figure 1.8; Table 1.1). PfHsp70-z lacks the linker domain, hence lacks allosteric control as compared to other PfHsp70 homologues (Vogel *et al*., 2006). Therefore, PfHsp70-z is believed to function as an NEF for PfHsp70-1 in the parasite cytosol (Shonhai *et al*., 2007; Muralidharan *et al*., 2012). *P. falciparum* knock-out studies have shown that PfHsp70-z is essential for parasite survival (Table 1.1; Muralidharan *et al*., 2012). Recently, PfHsp70-z was shown to exhibit chaperone activity by inhibiting the heat-induced aggregation of luciferase *in*

vitro (Zininga *et al.*, manuscript in preparation). Furthermore, PfHsp70-z was shown to cooperate with PfHsp70-1 to show an enhanced aggregation suppression activity of luciferase *in vitro* (Zininga *et al.*, manuscript in preparation).

1.5.2 *P. falciparum* Hsp70s localised to the endoplasmic reticulum

PfHsp70-2 or *P. falciparum* immunoglobulin heavy chain binding protein (PfBiP) is a stress-inducible molecule localised within the endoplasmic reticulum (Figure 1.8; Table 1.1; Kumar *et al*., 1991; Shonhai *et al*., 2007). Through electron-microscopy experiments, it was determined that PfBiP is primarily localised to the parasite ER (Kumar and Zheng, 1992). PfBiP shares 53% identity with PfHsp70-1 (Shonhai *et al*., 2007). Like other Hsp70 proteins, PfBiP can typically be induced by heat stress, but unlike eukaryotic Hsp70, PfBiP cannot be induced by glucose deprivation (Kumar *et al*., 1991). Ramya and colleagues demonstrated that PfHsp70-1 and PfBiP can inhibit the thermal unfolding of protein substrates. PfHsp70-1 and PfBiP's chaperone activities are both enhanced by the presence of PfHip (Ramya *et al.*, 2006). LaCount and co-workers (2005) showed that PfBiP interacts with erythrocyte *P. falciparum* membrane protein (Pf11803c), a protein that is exported from the parasite into the erythrocyte. PfBiP has also been described to physically interact with proteins destined for the erythrocyte during the intraparasite translocation phase (Saridaki *et al*., 2008). Heiny and co-workers (2012) have also suggested that PfBiP may be involved with trafficking apicoplast targeted proteins. PfBiP, therefore, plays an important role to export proteins of parasitic origin into the erythrocyte (Kumar *et al*., 1991; Vincensini *et al*., 2005; Saridaki., 2008).

PfHsp70-y and PfHsp70-z exhibit structural similarities that are distinct from the other PfHsp70 homologues. PfHsp70-y is similarly larger than other PfHsp70 homologues (Figure 1.8; Table

1.1). PfHsp70-y lacks a distinct linker domain similar to PfHsp70-z and also possesses an ER retention signal (Figure 1.7; Kumar *et al*., 1991; Lui and Houry, 2014). Thus, PfHsp70-y is believed to function as a NEF for PfBiP in the ER (Figure 1.6; Table 1.1; Vogel *et al*., 2006; Shonhai *et al*., 2007). Although, Heiny and colleagues (2012) showed that PfHsp70-y was detectable in the ER, apicoplast and parasitophorous vacuole. This study concluded that PfHsp70-y is unlikely to be a genuine apicoplast protein because the study could not localise the full length protein, only truncated fusions of the protein. Thus the localisation of the endogenous protein is unknown and its detection outside the confines of the ER may be a result of subcellular contamination (Heiny *et al*., (2012).

1.5.3 *Plasmodium falciparum* Hsp70 restricted to the mitochondrion

P. falciparum Hsp70 homologues are widely spread within the parasite cell. PfHsp70-3 possesses a mitochondrion targeting signal and is, therefore, thought to be restricted in the mitochondrion (Figure 1.7; Shonhai *et al*., 2007; Njunge *et al*., 2013; Lui and Houry, 2014). PfHsp70-3 has also been reported to be present in the PV (Nyawidhe and Lingelbach, 2006), however, this may also be a result of subcellular contamination.

1.5.4 *Plasmodium falciparum* Hsp70-x

Parasite-encoded hsps are thought to play a vital role in the in the trafficking of parasite-proteins to the erythrocyte and host remodelling (de Koning-Ward *et al*., 2008; Maier *et al*., 2009). PfHsp70-1 shares close similarities with PfHsp70-x the two homologues share a 73% sequence identity, higher than was observed for any other PfHsp70 homologues (Shonhai *et al*., 2007). PfHsp70-x was initially considered to be restricted in the cytosol but has recently been shown to

be exported to the erythrocyte (Külzer *et al*., 2012). PfHsp70-x is considered to facilitate parasite protein export and refolding in the erythrocyte. Furthermore, PfHsp70-x was shown to associate in complexes with exported PfHsp40 proteins in infected erythrocytes (Külzer *et al*., 2010; 2012).

The *P. falciparum* genome encodes fifty-one Hsp40 homologue proteins (reviewed in Njunge *et al*., 2013). Hsp 40s are classified into four types according to the presence of the J-domain; GF domain, zinc binding domain and a C-terminal (section 1.5.3). In *P. falciparum* eighteen of the Hsp40 were predicted to have the PEXEL sequence (Botha *et al*., 2007). Hence, some PfHsp40 proteins are thought to be exported into the infected erythrocyte cytosol. Maier and colleagues (2008) demonstrated that Type IV Hsp40 (PF10_0310) was essential for the pathogenicity of *P. falciparum*. The mutation/deletion of this gene, resulted in the loss of parasite-induced adhesive knobs on the surface of the infected erythrocyte (Maier *et al*., 2008). Hence, exported PfHs40 proteins are thought to facilitate the structural remodeling of the infected erythrocyte (Külzer *et al*., 2012). PfHsp70-x has been shown to co-localise with PfHsp40 homologues inside J-dots (Figure1.5). J-dots are mobile structures formed after the trophozoite stage, within the infected erythrocyte cytosol (Külzer *et al*., 2012). J-dot proteins contain type II Hsp40 proteins comprising a GF domain, thus can potentially interact with exported PfHsp70-x protein. J-dots are believed to partially co-localise with PfEMP1 in infected-erythrocytes. Hence, PfHsp70-x may be involved in protein translocation and *P. falciparum* pathogenicity of *P. falciparum* in the infected host cell, making it an attractive drug target (Pesce *et al*., 2010; Shonhai *et al*., 2010; Külzer *et al*., 2012). Moreover, since Hsp70 from *P. falciparum* are highly conserved they are

likely to evolve at a much slower rate compared to less conserved protein families. Therefore, are less likely to be susceptible to drug resistance (Edkins and Blatch, 2012).

1.6 Malaria heat shock proteins as potential drug targets

PfHsp60, PfHsp70-1, PfHsp90 and PfHop have all been shown to be upregulated in malaria cultures (Polpanich *et al*., 2007). Furthermore, this suggests that these molecular chaperones may have a common regulatory trigger and progression in the disease coincides with their expression. Furthermore, the expression of PfHsp70-1 has been shown to increase in response to anti-malarial drug pressure. HSPA1A and PfHsp70-1 are sufficiently unique to allow for selective inhibition (Cockburn, 2012). Hence, PfHsp70-1 could potentially be used as selective drug targets (Pesce *et al*., 2010; reviewed in Shonhai, 2010). Cockburn and co-workers (2011) showed that PfHsp70-1 and PfHsp70-x could potentially be targeted using malonganenone-A, small molecule antimalarial inhibitor. This inhibitor was found to interfere with the chaperone role of PfHsp70-1 and not HSPA1A, with significant toxicity towards *P. falciparum* as compared mammalian cells (Cockburn *et al*., 2011).

Recently a number of researchers have focused on natural compounds to alleviate the shortage of effective antimalarial drugs. Midorikawa and Haque (1997) showed that 15-deoxy-spergualin (DSG) is an immunosuppressive drug which has inhibitory effects on malaria parasite growth, both *in vivo* and *in vitro*. Ramya and co-workers (2007) suggested that DSG interact with PfHsp70-1 to inhibit the growth of *P. falciparum* parasites. This group showed that DSG interfered with the chaperone role of PfHsp70-1 in the export of nuclear-encoded proteins to the apicoplast, by interacting with the EEVD motif of PfHsp70-1 (Nadler *et al*., 1998; Ramya *et al*.,

2006). DSG is thought to interact with PfHsp70-1 via its C-terminal EEVD motif and subsequently precipitate PfHsp70-1, thereby interfering with the chaperone role of PfHsp70-1 (Ramya *et al*., 2007). Alternately, DSG may compete with the positively charged transit peptide destined for the negatively charged pores on the apicoplast membrane (Ramya *et al*., 2007). DSG thus prevents nuclear-encoded proteins to the apicoplast entry into the apicoplast, thereby inhibiting the growth of *P. falciparum* growth (Ramya *et al*., 2007). Banerjee and colleagues (2012) suggested that DSG may interfere with the interaction between PfHsp70-1 and *P. falciparum Enoyl-ACP Reductase* (PfENR). PfENR is a nuclear-encoded and apicoplast targeted protein, which is crucial for fatty acid biosynthesis in *P falciparum* parasites (Ralph *et al*., 2004; Smith *et al*., 2003). PfHsp70-1 facilitates PfENR translocation through the negatively charged pores in the apicoplast membranes. DSG interferes with the physical interaction between PfENR transit peptide and PfHsp70-1, as DSG will be bound to the EEVD motif of PfHsp70-1 (Banerjee *et al*., 2012). Other compounds structurally related to DSG, such as pyrimidinones, have also been described to interact physically with PfHsp70-1. Pyrimidinones have been shown to interfere with the chaperone activity of PfHsp70-1 and also inhibit the growth of *P. falciparum* parasite cultures (Botha *et al*., 2011; Chiang *et al*., 2009). Cockburn and colleagues (2011) identified novel classes of small molecule inhibitors of PfHsp70-1 and PfHsp70-x. In addition, they were found to inhibit the growth of *P. falciparum*-infected erythrocytes (Cockburn *et al*., 2011). Hsp70 and Hsp90 chaperones cooperate in activating signal transduction proteins by forming multi chaperone complexes (Pratt *et al*., 2003). Vaccine studies on *P. falciparum*-infected monkeys have shown immunity to PfHsp90 (Bonnefoy *et al*., 1994). These studies further demonstrate the validity of PfHsp70-1 as a potential drug target. The discovery of Hsp90, as a potential anti-cancer drug target, has led to insinuation that Hsps may play vital roles in the

induction and propagation of human diseases (Beliakoff and Whitesell, 2004). Hsp90 is, usually, associated with Hsp70, and are regulated by co-chaperones such as Hsp40, Hop, and Hsp interacting protein (Hip). The PBD of Hsp70 proteins has a highly conserved EEVD sequence motif (Freeman *et al*., 1995; Hartl *et al*., 2011). Hsp70 EEVD motif is vital for the formation of the Hsp70-Hsp90 complex structure through the binding of Hop (Odunuga *et al*., 2004; Gitau *et al*., 2012). Hernandez and colleagues (2002) demonstrated that Hsp40 enhanced the binding of Hsp70 to Hop. In malaria patients, PfHsp90 is highly expressed during parasite-induced fever episodes (Roy *et al*., 2012). PfHsp90 is considered to be essential to the survival of the parasite during the erythrocytic stage (Banumathy *et al*., 2003; Roy *et al*., 2012). PfHsp90 is known to regulate the transition from ring to trophozoite stage (Banumathy *et al*., 2003). PfHsp90 has also shown to be adequately different to human Hsp90 and allows for selective drug targeting (Pesce *et al*., 2010). Pallavi and co-workers (2010) found that PfHsp90 binds and hydrolyzes ATP more efficiently than human-hsp90. This group showed PfHsp90 binds to geldanamycin (GA; an Hsp90 inhibitor) with high affinity and consequently abrogates its chaperone function (Pallavi *et al*., 2010). Furthermore, GA was shown to inhibit parasite growth (Pallavi *et al*., 2010). Banumathy and colleagues (2003) demonstrated that GA arrests parasite growth from ring to trophozoite stage, highlighting PfHsp90 as a potential drug target. Hence, the understanding of PfHsp90 function is vital for inhibiting parasite growth and drug design.

1.7 Role of molecular chaperones in malaria immunology

The malaria parasites have adapted to survive under conditions prevailing in the warm-blooded host following their introduction from cold-blooded mosquito vectors (section 1.2.1). Interestingly the parasite manages to maintain its proteomic structural integrity under these

varied and harsh physiological conditions it encounters in the host. Furthermore, after infection febrile temperature episodes experienced by host, augment parasite development and infectivity (Pavithra *et al.*, 2004; Campos *et al*., 2010). The high temperatures associated with malaria fever should favour protein unfolding and aggregation (Ellis, 2000). However, the parasite has adapted to survive under these stressed conditions due to its expression of its molecular chaperones (section 1.4) (Zugel and Kaufmaan, 1999). It has been suggested that *P. falciparum* heat shock proteins (section 1.4.1) may assist the parasite survive the challenges it faces in the host by maintaining proteostasis (reviewed in Shonhai *et al*., 2011; Shonhai, 2014). *P. falciparum* parasites that are initially exposed to heat shock exhibit improved heat resistance, survival resilience and enhanced infectivity to subsequent heat treatments (Pavithra *et al.*, 2004; Cardoso *et al*., 2010). It has also been proposed that protein constituents from *P. falciparum* can modulate the function of immune cells, such as dendrite cells (DCs) (Urban *et al*., 2002).

The interference of DCs maturation by *P. falciparum* contributes to delayed antimalarial immunity (Urban *et al*., 2002). *P. vivax* infected erythrocytes have also been shown to modulate DCs maturation (Rigano *et al*., 2007; Sponaas *et al*., 2006; Wykes *et al*., 2007). Bettiol and colleagues (2010) demonstrated that DC maturation can be compromised in the presence of *P. yoelii*-infected erythrocytes (Bettiol *et al.*, 2010). Hence, molecular chaperones are believed to aid parasite infectivity (Shonhai, 2010; Shonhai *et al*., 2011). Hisaeda and co-workers (2005) proposed that certain molecules from infected erythrocytes interfere with the initiation of protective immunity after the infection. PfHsp70-1 may potentially modulate the host immune system (reviewed in Shonhai *et al*., 2011). Asea and co-workers (2003) proposed that molecular chaperones play a role as signal transducers (chaperokine function). It is thought that

chaperokines are capable of modulating immune cells, such as, monocytes, DCs, neutrophils and macrophages by binding to cell receptors (Asea *et al*., 2002; Asea, 2003). None-specific immune responses are triggered by the release of immune stimulatory molecules from lysed infected erythrocytes caused by replicating parasites (Hiaseda *et al.* 2005). Earlier studies demonstrated that Hsp70s can act as potent activators of the innate immune system and over-expression of hsp70 in certain organs may promote immunopathology. Thus suggesting may have inappropriate immune reactivity to hsp70 resulting in inappropriate pro-inflammatory responses and the development of autoimmune disease (Kaufman, 1990). Millar and co-workers (2003) suggested that during infection, an infected host loses the ability to differentiate between self and non-self-auto response, leading to inflammation and tissue destruction. Hsp60 proteins in mammalian cells have been demonstrated to be strong immune modulators, responsible for autoimmune and arthritic responses (Young *et al*., 1988). Sharma and colleagues (2012) reported clinical cases of patients who developed post malaria autoimmune mediated haemolysis. Polpanich and colleagues (2007) reported that some *P. falciparum* Hsps were detectable in the blood-stream. Thus, it is likely that *P. falciparum* Hsps that leak in the bloodstream of infected individuals may promote the development of autoimmune diseases. However, other reports have shown that the administration of recombinant heat shock protein 70 (hsp70) can attenuate experimental autoimmune disease (Wendling, 2000). Thus further studies are required to elucidate the immunomodulatory role of Hsps.

1.8 Toll-like receptors are vital in immunomodulation

Toll-like receptors (TLRs) are conserved pattern recognition receptors found on the on immune cells (Gearing, 2007). TLRs are known to recognise distinct microbial ligands, allowing the host

cells to distinguish between host and non-self molecules (Takeuchi and Akira, 2010). TLRs are readily activated pathogen-associated molecular patterns (PAMPs). PAMPs are non-self molecules containing lipids, lipopeptides, proteins, and nucleic acids from a microbial source (reviewed in Kawai and Akira, 2007). TLR activation induces innate immune response and primes antigen-specific adaptive immunity cells (Gearing, 2007). TLRs are, therefore, pivotal in the establishment of the innate and adaptive immune system. Currently eleven human TLRs have been identified, each recognising different PAMPs derived from various microorganisms such as bacteria, viruses, protozoa and fungi (Akira *et al*., 2006). TLRs generally operate in complexes with other TLRs to recognise specific ligands. TLR2 is believed to function hand in hand, with TLR1 or TLR6 to recognise peptidoglycan, lipopeptides and lipoproteins derived from Gram-positive bacteria. Gram-negative bacterial cell wall components such as lipopolysaccharides (LPS) (also referred to as endotoxins in this report) are recognised by TLR4 in complex with MD-2. Bacterial flagellins are detected by TLR5, whereas, nucleic acids are detected by TLR3, 7, 8 and 9 (Akira *et al*., 2006; Park and Lee, 2013). TLR1, 2, 4, 5, 6 and 11 are localised on the surface of the cell membrane, whereas TLRs recognising nucleic acids are restricted to cytoplasmic compartments. TLR-induced stimulation by PAMPs triggers intracellular signalling results in the expression of various inflammatory cytokines. TLRs can also be stimulated by host-derived molecules known as damage-associated molecular patterns (DAMPs) (Bianchi, 2007). DAMPs can be proteins produced from damage tissues, necrotic cells or proteins produced in response to inflammatory stimuli (Bianchi, 2007).

Aggregated or misfolded human antibodies have been shown to be immunogenic when administered to humans (den Englesman *et al.*, 2011; Singh, 2011). Joubert and colleagues

(2012) proposed that partially misfolded antibodies may also trigger an early innate immune response. According to Hermeling and colleagues (2005), the immunogenicity of proteins depends on the structure and orientation of the constituent protein molecules and/or on the aggregate size. Joubert and colleagues (2012) demonstrated that misfolded proteins may stimulate the production of pro-inflammatory and immunosuppressive cytokines. Protein aggregates are thought to modulate cytokine expression by binding to TLRs on the cells surface immune cells (Joubert *et al*., 2012). During the malaria cycle, after the schizonts mature, they rupture and release merozoites into the bloodstream (section 1.2.1; Wirth, 2002). Along with the merozoites are parasite proteins and Hsps that were exported into the host cell, which in the absence of molecular chaperones are likely to aggregate and may acts as DAMPs. Hsps, such as Hsp70 proteins have been suggested to function as a DAMPs stimulating cytokine expression on immune cells (Aosai *et al*., 2006, Floto *et al*., 2006; Carabarin-Lima *et al*., 2011; Luong *et al*., 2012).

1.9 Hsp70 proteins inducing the immune system

Apart from their role as molecular chaperones (section 1.5), it is believed that some parasite-derived Hsp70 proteins are capable of modulating host immunity through signal transduction (chaperokine role). Recently, Hsp70 has been shown to function as a TLR4 ligand (Zhang *et al*., 2013). Moreover, that in the absence of a TLR4 or TLR4 adapter molecule; the interaction between Hsp70 and TLR4 was nullified (Zhang *et al*., 2013). Fang and co-workers (2011) showed that Hsp70 might bind directly to TLR4 on the surface of DCs, thereby stimulating DCs to secrete proinflammatory cytokines through the TLR4 signalling pathway. *Toxoplasma gondii* is an obligate intracellular parasite that shares close homology to *P. falciparum* infection

(reviewed Shonhai *et al*., 2012). Hsps from *T. gondii*, have also been documented as potent immune activators (Mun *et al*., 2000). Hwang and co-workers (2010) showed the overproduction of *T. gondii* Hsp70 (TgHsp70) is anti-apoptotic and directly modulates the differentiation of DCs (Kang *et al*., 2004). The production of nitrogen oxide (NO) in cells has been reported to have an anti-parasitic effect (Bogdan *et al.*, 2001; Safeukui *et al*., 2008). Interestingly, TgHsp70 has been reported to inhibit NO production in macrophages, thereby, modulating the protective immune responses against infection by *T. gondii* (Dobbin *et al*., 2002; Mun *et al.*, 2000; Yano *et al*., 2002). Floto and colleagues (2006) showed that mycobacterial Hsp70 promotes DC aggregation, immune synapse formation between DCs and T cells. Initially, the NBD of Hsp70 proteins (section 1.4.2) were thought not to be necessary for the proteins immunomodulatory activity. Instead, it was believed that the C-terminal PBD of Hsp70 was essential for cytokine induction by the chaperone (Mun *et al*., 2000; Wang *et al*., 2002). However, Planelles and colleagues (2002) showed that *Trypanosome cruzi* Hsp70 NBD successfully induced DC maturation. Carabarin-Lima and co-workers (2011) presented evidence that the *T. cruzi* Hsp70 NBD had immunomodulatory role, although it was not as effective as a full-length Hsp70 protein.

Hsp70 chaperones have also been shown to interact with various peptides and proteins, regardless of their origin. Antigen peptides can be bound and delivered to MHC class I and II molecules by Hsp70, to modulate the immune system (Chicz *et al.*, 1992; van Eden, 2005). These Hsp70-peptide complexes have been shown to be critical to activating MHC-I, molecules present on the cell surface (Srivastava, 2008). Hsp70-peptide complex 96 vaccine has been reported to be effective in treating melanoma and kidney cancer (Testori *et al*., 2008). Hsp70–peptide complexes presented to MHC I and II molecules, have been demonstrated to induce an

enhanced antigen-specific T-cell activation (Udono and Srivastava, 1993; Tobian *et al*., 2004). Haug and co-workers (2007) proposed that proteins bound to Hsp70 may stimulate cell receptors through the peptide binding site of the Hsp70 molecules, hence, modulating the immune cells. Most investigations focusing on the chaperokine functions of Hsps use recombinant forms of the proteins produced in *Escherichia coli* expression systems. However, the main drawback of this expression system is that the produced recombinant protein co-purifies with endotoxins such as, LPS (section 2.1).

LPS are endotoxic molecules, which tend to co-purify with proteins produced in Gram-negative expression hosts (Konsman *et al*., 2002). Immunomodulatory studies based on the use of endotoxin contaminated proteins have confounding data. Bendz and co-workers (2008) showed that highly purified Hsps could not induce the production of proinflammatory cytokines. Similarly, Gao and Tsan (2005) demonstrated through gene expression arrays that Hsp60 and Hsp70 did not have cytokine stimulatory activity on macrophages. Therefore, whether Hsps have immunomodulatory roles is still an ongoing debate.

Other scholars have suggested that the synergistic interaction between Hsps and LPS was responsible for the exhibited immunomodulatory stimulation (Osterloh *et al*., 2007; Loung *et al*., 2012). Due to the fact that Hsps are responsible for folding nascent proteins and generally bind to molecules with exposed hydrophobic patches, they potentially bind LPS since LPS is a hydrophobic molecule. Hsp60 has been shown to bind directly to LPS, *in vitro* (Habich *et al*., 2005). Furthermore, Hsp60 and Hsp90 have been shown to bind strongly to LPS, thereby enhancing immunostimulating effects of LPS (Warger *et al*., 2006; Osterloh *et al*., 2007; 2008).

Osterloh and colleagues (2007) further showed that LPS bound to Hsp was more rigours than LPS alone in producing cytokine stimulation. In addition, it has been proposed that during Gram-negative bacterial infections, Hsps may bind to endotoxins thereby modulating the host defence system (Osterloh *et al*., 2007; 2008). However, currently there is no direct evidence showing that Hsps bind and interact with LPS, *in vivo*. Wang and co-workers (2006) showed that LPS-free Hsp70, purified using endotoxin-free plastic-ware and glass-ware successfully stimulated cytokine-production on macrophage cells. Parasite Hsp such as PfHsp70-1 have been reported to be present in the bloodstream of malaria infected individuals (Polpanich *et al*., 2007; Guirgis *et al*., 2012). Hence, it is plausible that if PfHsp70-1 leaks into host circulatory system, it may possibly invoke an immunogenic response (Kumar *et al*., 1990; Shinnick, 1991). Further studies are required to elucidate the role of PfHsp70-1 on immune cells.

1.10 Problem Statement and motivation

PfHsp70-1 is crucial for the survival of the malaria parasite (Shonhai *et al*., 2010). Zhang and co-workers (2001) found that the antibodies PfHsp70-1 and PfHsp65 were significantly increased in patients with malaria. This finding suggests that malaria Hsps may trickle into the blood-stream and can potentially modulate the immune system, as previously shown by Hsp from or intracellular parasites (Polpanich *et al*., 2007; Guirgis *et al*., 2012). Most studies focusing on the chaperokine function of Hsps use recombinant forms of proteins produced in *E. coli*. Interestingly, some of these studies investigating the chaperokine role of Hsp70 have proposed that the signalling action of Hsps may be confounded by the inherent endotoxin contamination (Gao and Tsan, 2003). Since most recombinant proteins are expressed in *E. coli*, LPS co-purifies with the protein during the purification stage (Bausinger *et al*., 2002; Gao and Tsan, 2003). The

current study sought to resolve this controversial data by producing LPS-free recombinant protein using various bacterial protein expression hosts.

1.11 Research Hypothesis

Parasite Hsp70s have been implicated as immunomodulators. The identification of antibodies to PfHsp70-1 in malaria patients (Kappes *et al*., 1993; Pallavi et al., 2010) suggests that PfHsp70-1 may have an immunomodulatory role. Hence, the current study sought to investigate the effect of PfHsp70-1 on innate immune cells.

1.12 Broad objectives

The specific objectives of the study were to:

1) Express and purify recombinant PfHsp70-1 protein using *E. coli* XL1 Blue, *E. coli* Clearcoli BL21 and *Brevibacillus choshinensis*
2) Clone, express and purify recombinant PfHsp70-1 nucleotide binding domain
3) Compare the biological activity of PfHsp70-1 produced from *E. coli* Clearcoli BL21 and *Brevibacillus choshinensis*
4) Conduct cytokine induction assays using PfHsp70-1

CHAPTER 2

EXPRESSION AND PURIFICATION OF RECOMBINANT PFHSP70-1

2.1 Introduction

Heterologous protein expression using the Gram-negative bacterium, *E. coli*, is an important and frequently used tool within malaria research for expressing recombinant proteins. Bacterial expression systems are widely used and favoured by industrial and pharmaceutical companies, because of their ability to rapidly produce recombinant proteins, using low cost substrates (Góes *et al*., 2010). Nonetheless, this approach is associated with many challenges. Most proteins including those from *Plasmodium* are expressed as insoluble proteins in inclusion bodies (Mehlin *et al*., 2006). Inclusion bodies primarily contain over-expressed non-native proteins and occurs as a result of differences between the codon usage of the *E. coli* and the protein of interest (Singh and Panda, 2005; Terpe, 2005; Gopal and Kumar, 2013). Mehlin and colleagues (2006) selected and attempted to express 1000 *P. falciparum* open reading frames using *E. coli* BL21 Star^{TM} (DE3) cells, of these only 6.3% where expressed as soluble proteins. The same group attempted to improve expression by a technique called "codon-optimisation" an approach where codons of the target gene are altered to suit the codon preference of expression host (Zhou *et al*., 2004). Codon-optimisation had no significant influence on the production of *P. falciparum* proteins from *E. coli* (Mehlin *et al*., 2006). Consequently, less than 0.5% of all protein structures available in Protein Data Bank are malarial proteins (Carrio and Villaverde, 2002; Flick *et al*., 2004). The production of functional malaria proteins is vital for vaccine studies and antimalarial research (Birkholtz *et al*., 2008).

Recombinant proteins purified from the *E. coli* expression system tend to co-purify with endotoxin such as lipopolysaccharides (LPS) attached to them. LPS (endotoxins) are key component of the outer membrane of Gram-negative bacteria, responsible for the stability of the bacterial membrane (Fiske *et al*., 2001; Buttenschoen *et al*., 2010). LPS are known to

induce a severe immune response, in humans (Mader *et al*., 2004). *E. coli* LPS is composed of three distinct regions: the O-antigen, a core oligosaccharide and lipid A. Lipid A is the most conserved part of LPS, and is responsible for most biological endotoxic activity (Buttenschoen *et al*., 2010; Petsch and Anspach., 2000) and it is recognized as a PAMP by TLR4 on immune cells and stimulates them to secrete pro-inflammatory cytokines. LPS is, however, pyrogenic to humans and if introduced into the blood stream of human or other mammals, it can cause severe tissue failure. At high concentrations LPS can induce the overproduction of cytokines, resulting in autoimmune diseases or even the activation of non-specific innate immune cells and potentially lethal septic shock (Konsman *et al*., 2002; Raetz, and Whitfield, 2002). Techniques such as affinity chromatography have been shown to be effective in removing LPS contaminating recombinant proteins (Petsch and Anspach, 2000). These methods, however, do not completely remove the contaminants; leading to confounding data as LPS is a strong immune activator (Gao and Tsan, 2003). Moreover, these techniques are believed to reduce the biological activity of the proteins being purified (Minawaga *et al*., 2012).

Other systems that have been widely recommended as LPS-free protein expression systems are mammalian cells and baculovirus-infected insect cells (Geisse & Henke, 2005; Possee *et al*., 2008). Although these two systems have been widely used for LPS-free expression of recombinant proteins, they are very laborious, time-consuming and difficult to maintain (Possee *et al*., 2008). Recently a private company, Lucigen (Germany), launched the *E. coli* ClearColi™ BL21 (DE3) protein expression cells with a modified LPS (Lipid IV_A), comprise of four acyl chains instead of the six acyl chains in normal *E.coli* LPS (Figure 2.1; Mamat *et al*., 2013). The LPS mutation is thought to disable the endotoxin immune response. LPS has previously been shown to lose immune stimulatory activity either when it lacks one or has

more than six acyl chains (Park and Lee, 2013). The six acyl chains in LPS are recognized by Toll like receptor-4 (section 1.8) in complex with myeloid differentiation factor 2 (MD-2), resulting in the production of proinflammatory cytokines. The deletion of the two secondary acyl chains results in lipid IV_A, which does not induce the secretion of proinflammatory cytokines. Furthermore, LPS from *E. coli* ClearColi BL21 cells also habour a deleted oligosaccharide chain to further facilitate the removal of the residual LPS on the downstream product, if required (Figure 2.1; Mamat *et al*., 2013).

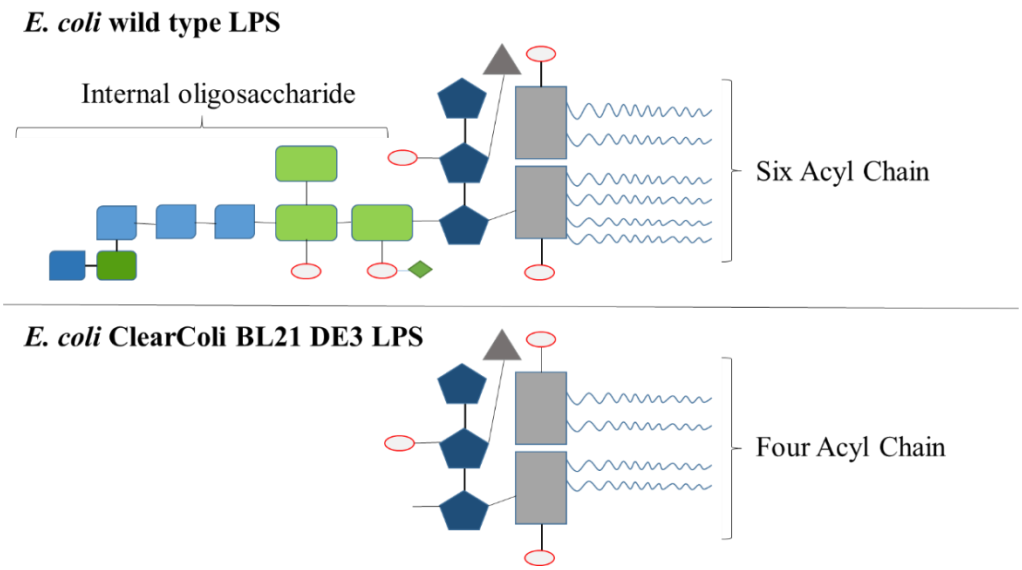


Figure 2.1 Structural comparison of normal LPS with genetically modified ClearColi LPS
LPS from wild type *E. coli* has six acyl chains vital for immunogenic stimulation. Whereas, in *E. coli* ClearColi BL21 cells the internal oligosaccharide chain is deleted, and two of the six acyl chains are removed to disable the endotoxin signal.

Most pharmaceutically produced proteins such as cytokines (Shiga *et al*., 2000) and enzymes (Kashima and Udaka, 2004; Malten *et al*., 2005) have previously been produced using Gram-positive expression strains. *Bacilli* strains are attractive hosts due to their ability to express heterologous protein inside cultured cells and secrete the expressed protein into the extracellular medium (Westers *et al*., 2004; D'Urzo *et al*., 2013). The *Bacilli* expression system thus offers an attractive alternative to the problematic expression of high protein

expression levels in *E. coli.* Takara-Bio (Japan) also launched an LPS-free protein expression system, *Brevibacillus* protein expression System II (D'Urzo *et al*., 2013). This expression system employs Gram-positive bacterium, *Bacillus brevis* (*Brevibacillus*) *choshinensis* (Takara-Bio, Japan). This strain has mutated SP3 gene required for spore formation. The *B. choshinensis* strain also has a mutated intracellular protease gene (imp) and extracellular protease gene (emp) to further protect the structural integrity of expressed protein. Currently, there is no data suggesting the reliability of these bacterial cells to produce LPS-free malaria proteins. Hence, the broad objective of this study was to express *P. falciparum* Hsp70-1 using *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* expression hosts.

E. coli XL1 Blue cells are endonuclease (endA) and recombination (recA) deficient, which greatly improves their stability to retain DNA plasmid. The successful expression and purification of various *P. falciparum* Hsps have previously been documented using protein expression hosts such as *E. coli* XL1 Blue (Matambo *et al*., 2004; Cockburn, 2012; Gitau *et al*., 2012). *P. falciparum* Hsps play an important cytoprotective role, ensuring that the malaria parasite survives under the harsh conditions that prevail in the host environment (section 1.5; Shonhai *et al*., 2010). Recombinant PfHsp70-1 (Matambo *et al*., 2004; Ramya *et al*., 2006; Shonhai *et al*., 2008; Misra and Ramachandran, 2009), PfHsp70-2 (Ramya *et al*., 2006), PfHsp70-z (Zininga *et al*., manuscript in preparation), PfHsp40 (Botha *et al*., 2011), PfHop (Gitau *et al*., 2012) and PfHsp90 (Pallavi *et al*., 2010) have all been successfully produced using *E. coli* as expression hosts. PfHsp70-1, which is the focus of this study, has previously been purified from an *E. coli* XL1 Blue host (Shonhai *et al*., 2008) and are likely to co-purify with LPS with LPS contamination. Certain parasite Hsps such as *T. gondii* Hsp70 have been deemed as immunomodulatory (Mun *et al*., 2000). For example *T. gondii* Hsp70 has been shown to induce cytokines expression on immune cells (Hwang *et al*., 2010). However,

contention exists that the above findings may be due to the production of Hsps using an *E. coli* host. Habich and co-workers (2005) showed that Hsp60 specifically binds to bacterial LPS. It is thus, conceivable that LPS may also be introduced by peptides co-purified along with PfHsp70-1. Due to the chaperone role of PHsp70-1 (section 1.5.1). Since, Hsp70 proteins potentially bind to hydrophobic residues, thereby, LPS is potentially bound by the PBD. It is therefore important to produce recombinant Hsps using methods or expression hosts that exclude LPS contaminants.

Methods used to remove LPS contaminating from recombinant proteins, are also believed to interfere with the biological activity of the proteins (Petsch and Anspach, 2000). The ATPase basal activity can be used to compare the biological activity between proteins, *in vitro*. Biological assays have been developed to determine Hsp70 basal ATPase activity through the detection of inorganic phosphate, released when ATP is hydrolysed (Chifflet *et al*., 1988). ATP-bound Hsp70 displays an open state of the PBD, were substrates are rapidly bound and released by Hsp70 (Brinker *et al*., 2002). The hydrolysis of ATP results in closing of the PBD and subsequent reduction in Hsp70 affinity for substrates (section 1.4.2; *Fan et al*., 2003). The ATPase activity of PfHsp70-1 has been demonstrated to be significantly higher than both human and bacterial Hsp70s (Matambo *et al*., 2004; Shonhai, 2007). Recombinant proteins are sensitive to various stages of expression and purification, which can result in modifications of protein structure leading to protein aggregation and reduced activity (Talarico *et al*., 2005).

The study, therefore, sought to heterologously express and purify PfHsp70-1 excluding LPS contaminants. Furthermore, a comparative study was conducted to determine the biological activity of PfHsp70-1 produced in *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B.*

choshinensis. To determine the PfHsp70-1 domain that binds to LPS. *P. falciparum* lactate dehydrogenase (PfLDH) is a malarial protein has no known chaperone activity, PfLDH is an ideal negative control as it has no known chaperokine activity. Hence, PfLDH has been included in this study to enable comparative immunomodulatory analysis.

The objectives of the study were to:

1) Express and purify PfHsp70-1 in *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensi.*
2) To determine levels of LPS associated with purified recombinant PfHsp70-1.
3) Determine the ATPase activity of recombinant PfHsp70-1.

2.2 Experimental procedures

2.2.1 Materials

The following plasmids and reagents were used: pQE30 vector (Qiagen, Germany) and pQE30/PfHsp70-1 plasmid expressing PfHsp70-1 (Table 2.1; Matambo *et al*., 2004; Shonhai *et al*., 2005; Gitau, 2014) and the plasmids were extracted using ZR Plasmid Miniprep™ kit (Epigenetics, U.S.A). PfLDH encoding plasmid was a kind donation from Prof. Dean Goldring of the department of Biochemistry, University of KwaZulu Natal, South Africa (Table 2.1). Bacterial growth media and buffers were made using LPS-free package plastic ware, baked glassware (250°C for 4 hours) and HyPure™ Cell Culture Grade water (HyClone, U.S.A) to minimise LPS contamination. The rest of the reagents used in the study are listed in (Appendix C; Table C.1).

Table 2.1 Description of strains and plasmids used in this study

Strains & plasmids	Description	Supplier/Reference
Strains		
E. coli XL1 Blue	recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lac [F´proAB lacIqZM15 Tn10 (Tetr)].	Bullock *et al*., 1987
E. coli ClearColi™ BL21	msbA148 gutQ kdsD lpxL lpxM pagP lpxP eptA	Mamat *et al*., 2013
B. choshinensis	(Genotype unknown, supplier will not disclose)	Takagi *et al*., 1993
Plasmids		
pQE30-PfHsp70-1	pQE30 encoding PfHsp70-1, Amp^R	Shonhai *et al*., 2008
pQE30/PfHsp70-1_{NBD}	pQE30 encoding PfHsp70-$1_{NBD,}$ Amp^R	This Study
pNI-His/PfHsp70-1	pNI-His encoding PfHsp70-1, Amp^R	This Study
pK223-3/PfLDH	pK223-3 encoding PfLDH, Amp^R	Krause, 2011

2.2.2 Confirmation of pQE30/PfHsp70-1 plasmid construct encoding PfHsp70-1 protein

The pQE30/PfHsp70-1 construct (Matambo *et al*., 2004; Shonhai *et al*., 2008) was used to express PfHsp70-1. To confirm the integrity of the pQE30/PfHsp70-1 plasmid construct, the plasmid was purified using the ZR Plasmid Miniprep™ kit (Epigenetics, U.S.A) according to manufactures instructions (Appendix A.3). The DNA was digested (Appendix A.4) using restriction enzymes, *HindIII* and *BamHI* (Thermo Scientific, U.S.A). The restriction products were then analysed using agarose gel electrophoresis (Appendix A.5).

2.2.3 Construction of plasmid expressing PfHsp70-1$_{NBD}$

The interaction between Hsp70 and lipopolysaccharides or lipids is well documented, however, the residues involved in this interaction are still not known (Arsipe *et al*., 2002; McCallister and Nikolaidis, 2014). This study therefore cloned the construct expressing the subdomain of PfHsp70-1, excluding the C-terminal peptide binding domain of the protein. The DNA construct expressing full-length PfHsp70-1 (pQE30/PfHsp70-1) which was previously used to express the protein in *E. coli* (Shonhai *et al*., 2008), was used as a template and primers were designed to place a stop codon immediately downstream of the bases encoding for the residue S398 which marks the end of the ATPase domain of PfHsp70-1. The Quick-change II Site-directed Mutagenesis Kit (Stratagene, U.S.A) was used to introduce the stop codon on the plasmid following the standard provided by the manufacturer. The following primers were used: forward primer: 5′-GGTGACCAATCA**TAA**CTAGTCCAAGATTTATTATTATTAG-3′; and the reverse primer: 5′- CTAATAATAATAAATCTTGGACTAG**TTA**TGATTGGTCACC-3′ (stop codon in bold). A *Spe*I restriction site (underlined) was added downstream of the stop codon. The following PCR parameters were used; Initial denaturation, (95°C for 1 minute), 18 cycles of denaturation (95°C for 50 seconds), annealing (60°C for 50 seconds) and elongation (68°C

for 5.4 minutes). The successful insertion of the stop codon was confirmed by digestion of the construct using *BamHI* and *SpeI* (Thermo Scientific, U.S.A). DNA was resolved using agarose gel electrophoresis coupled with DNA sequencing to verify the integrity of pQE30/PfHsp70-1NBD.

2.2.4 Confirmation of pKK223-3/PfLDH encoding PfLDH protein

The pKK223-3/PfLDH construct (Krause, 2011) was used to express recombinant PfLDH. A restriction digest using *PstI* and *EcoRI* (Thermo Scientific, U.S.A) and DNA sequencing analysis was performed to confirm the integrity of the pKK223-3/PfLDH plasmid construct.

2.2.5 Cloning strategy of pNI-His/PfHsp70-1 towards expression and purification

The *Brevibacillus* expression system utilises a pNI-His vector equipped with a P2 protein expression promoter as compared to the pQE30 vector (T5 promoter) used in *E. coli* cells (Table 2.1). Taking advantage of the common *BamHI* and *HindIII* restriction sites on the multiple cloning sites of the pNI-His and pQE30 vectors, PfHsp70-1 from pQE30/PfHsp70-1, was gel-purified using the Zymoclean™ gel DNA Recovery Kit (Epigenetics, U.S.A), following the manufacturer's instructions. The PfHsp70-1 DNA segment was then ligated with a gel purified pNI-His vector, which had already been digested with *BamHI* and *HindIII*, yielding the pNI-His/PfHsp70-1 plasmid. Restriction digest and DNA sequencing analysis were used to confirm the integrity of the pNI-His/PfHsp70-1 plasmid.

2.2.6 Expression of recombinant proteins in *E. coli* XL1 Blue and *E. coli* ClearColi BL21 DE3 competent cells

Competent *E. coli* XL1 Blue and *E. coli* ClearColi BL21 DE3 cells where chemically transformed with PfHsp70-1, PfHsp70-1_{NDB} and PfLDH (Appendix A.2; Shonhai *et al.*, 2008). The colonies carrying the plasmids were respectively grown for 12 hours in 500 ml

yeast/tryptone (YT) broth containing 100 µg/ml ampicillin, then subsequently diluted into 450 ml of Terrific broth (Tryptone 12 g/L, Yeast extract 24 g/L, Glycerol 4 mL/L, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4), an auto induction media containing 100 µg/ml ampicillin overnight. The cultures were allowed to grow at 37°C with agitation at 160 rpm. The protein expressing cells were harvested, by centrifugation (5 000 rpm, 20 min at 4°C) and thereafter resuspended in 10 ml of non-reducing lysis buffer (10 mM Tris, pH 7.5, 300 mM NaCl and 10 mM imidazole) containing 1 mM phenylmethylsufonyl fluoride (PMSF) and 1 mg/ml of lysozyme. The cells were kept at -80°C for storage. Protein expression samples were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie blue. The production of the PfHsp70-1 was confirmed by Western blot (Appendix A.9) using rabbit-raised polyclonal anti-PfHsp70-1 [α-PfHsp70-1 (1:2000 dilution)], with a goat raised anti-rabbit horseradish peroxidase conjugated antibody (1:2000 dilution), used as the secondary antibody. The production of the His_6-tag on the recombinant proteins was confirmed using mouse monoclonal anti-His_6-Peroxidase antibodies [α-His (1:2000 Dilution)] (Thermo Scientific, U.S.A). Western blot images were acquired using CL-Xposure™ X-ray film (Thermo Scientific, U.S.A).

2.2.7 Expression of recombinant PfHsp70-1 in *B. choshinensis*

The *Brevibacillus* expression system allows extracellular and intracellular protein expression without LPS contamination (D'Urzo *et al*., 2013). PfHsp70-1 cloned into the pNI-His vector, encoding an N-terminal His_6-tag, was used to facilitate PfHsp70-1 production using the *B. choshinensis* cells (Takara-bio, Japan). Competent *B. choshinensis* cells where transformed with pNI-His/PfHsp70-1 plasmid following manufacturer's protocol. Colonies carrying the pNI-His/PfHsp70-1 plasmid were then inoculated into 500 ml MT broth (polypeptone 10 g/L, yeast extract 2 g/L, meat extract 2 g/L, glucose 10 g/L, $FeSO_4$ 10 mg/L, $MnSO_4$ 10 mg/L,

$ZnSO_4$ 1 mg/L and $MgCl_2$ 4.1 g/L [pH 7.0]), and incubate at 30°C (160 rpm) overnight. This study also compared the expression of intracellular PfHsp70-1 production against the extracellular expression. Colonies carrying the pNI/PfHsp70-1 construct where inoculated into 20ml MT broth. Cells (100ul) were harvested (5 000 g, 20 min at 4°C) after 24hrs, 48 and 64 hours protein expression and resuspended in 10 ml of non-reducing lysis buffer supplemented with 1 mM PMSF. The cells were then stored at -80°C. PfHsp70-1 expression was analysed on a SDS-PAGE gel and Western blot analysis was used to confirm the production of PfHsp70-1 and the production of the His_6-tag on the expressed protein.

2.2.8 Purification of PfHsp70-1, PfHsp70-1_{NBD} and PfLDH recombinant proteins

The *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* cell cultures used to express PfHsp70-1, PfHsp70-1_{NBD} and PfLDH stored at -80°C were allowed to thaw on ice. Polyethyleneimine (PEI) has been reported to aid the solubilisation of insoluble proteins, by precipitating nucleic acids complexed to the proteins leaving the target protein in the soluble fraction (Shonhai *et al*., 2008; Marenchino *et al*., 2009). Cells were thawed in the presence of 0.1 (v/v %) PEI to maintain the target proteins in a soluble and native state during purification (Shonhai *et al*., 2008; Marenchino *et al*., 2009; Gitau, 2014). The cells were then mildly sonicated at amplitude setting of 30 for seven cycles (15 seconds pulse and 5 seconds pause after each cycle). After sonication, the cell lysates were centrifuged at 5 000 rpm for 20 minutes at 4°C and the supernatant separated from the pellet. The supernatant containing the target recombinant proteins were suspended in 50% (w/v) slurry of nickel-charged HisPur™ Nitrilotriacetic acid (Ni-NTA) (ThermoScientific, U.S.A) for 1 hour with mild agitation at 4°C. The unbound protein was removed by washing the beads twice in wash buffer (300 mM NaCl, 150 mM imidazole, 10 mM Tris, pH 7.5). The bound His_6-tag proteins were eluted in 3

ml of elution buffer (300 mM NaCl, 1 M imidazole, 10 mM Tris, pH 7.5). The purified protein was extensively dialysed against dialysis buffer (300 mM NaCl, 10 mM Tris, pH 7.5) to remove imidazole using pre-soaked Slide-A-Lyzer® dialysis cassettes (Thermo Scientific, U.S.A). Aliquots of purified proteins were analysed by 12% SDS-PAGE and Western blot analysis was used to determine the presence of the protein of interest. Protein concentration was determined using Bradford's assay as described (Appendix A. 10). The purified protein was subsequently stored in lyophilised form.

2.2.9 Removal of LPS from PfHsp70-1 from *E. coli* XL1 Blue

The greatest drawback in immune studies using protein preparations produced from traditional *E. coli* expression systems has been LPS contamination (Gao and Tsan, 2003). To ascertain the LPS contamination status of the proteins expressed in this study, equal amounts of PfHsp70-1 purified from *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* cells were analysed for LPS contamination at various concentrations (0.1, 1 and 10 µg/ml) using the Limulus Amebocyte Lysate (LAL) Endo-toxate kit (Sigma Aldrich, U.S.A) according to manufacturer's recommendations.

2.2.10 The effect of ATP on PfHsp70-1-LPS association during recombinant purification

Hsp70 binds to unfolded proteins forming stable complexes that dissociate upon binding and hydrolysis of ATP (Shonhai *et al*., 2008). This study hypothesized that the release of these bound proteins from PfHsp70-1 may reduce the amount of LPS co-purified with protein expressed using *E. coli* XL1 Blue cells. Therefore, it is critical to determine the LPS content on PfHsp70-1 purified in the presence and absence of ATP. Protein purification was followed was as earlier stated (section 2.2.8) with minor alterations. Modifications included were in the

second wash, where one purification batch was supplemented with 10 mM ATP (Roche, Germany) and the other purification batch was not supplemented with ATP. The proteins were extensively dialysed to remove imidazole and ATP. The purified proteins from ATP supplemented and non-supplemented purification batches were allowed to flow through an Endotrap Red coloumn (Hyglos GmbH, Bernried, Germany) to remove LPS following manufacturer's instructions. The LPS content of the PfHsp70-1 protein batches were then compared using the EndoLISA kit (Hyglos GmbH, Bernried, Germany).

2.2.11 Biological activity assay to determine the functional status of PfHsp70-1 purified without LPS contamination

Gram-positive protein expression systems have been reported to produce proteins with a higher enzymatic activity compared to those expressed in *E. coli* (Talarico *et al*., 2005). The basal ATPase activity for PfHsp70-1 was determined using a previously described method with slight modifications (Chamberlain and Burgoyne, 1997; Matambo *et al*., 2004). The ATPase activity assays were performed using 0.4 µM PfHsp70-1 (purified from each of the three bacterial sources) in a buffer containing 10 mM Hepes, 100 mM KCl, 2 mM $MgCl_2$ and 0.5 mM dithiothreitol, pH 7.5. The reaction mix was pre-incubated at 37°C for 5 minutes, after which the reaction was initiated by the addition of ATP to a final concentration of 1 mM. The assays were performed at 37°C for 4 hours before stopping the reaction with 10% SDS and using 1.25% ammonium molybdate and 9% ascorbic acid to quantitate the release of inorganic phosphate. The reaction mix without the protein was used as a non-enzymatic control. Samples were read at 630 nm on a 96-well plate reader (Biotek Instruments, U.S.A). The absorbance values were extrapolated against a standard calibration curve using Na_2HPO_4 as standard. The basal ATPase activity was expressed as nmol Pi released/min/ mg of PfHsp70-1 protein.

2.2.12 Statistical Analysis

Differences in LPS contamination levels the experimental groups were evaluated by one-way ANOVA. Values of $P<0.05$ were considered statistically significant. The error bars represent estimated standard deviations calculated using Microsoft Excel, Version 11.2.3 and GraphPad Prism 6. All diagrams in this study are representative of data from three independent experiments.

2.3 Results

2.3.1 Confirmation of pQE30/PfHsp70-1 plasmid encoding PfHsp70-1

Restriction digest of pQE30/PfHsp70-1 was carried out to verify the integrity of the construct using *BamHI* and *HindIII* (Figure 2.2; Matambo *et al*., 2004). Digestion at the single restriction site with either *BamHI* or *HindIII* resulted in the migration of linearised plasmid of 5471 bp (Figure 2.2 B). Excision of the PfHsp70-1 coding sequence (2052 bp) was achieved through double digestion using *BamHI* or *HindIII* resulting in fragments of 3419 bp and 2052 bp, corresponding to the pQE30 expression vector and PfHsp70-1 insert sizes, respectively (Figure 2.2 B).

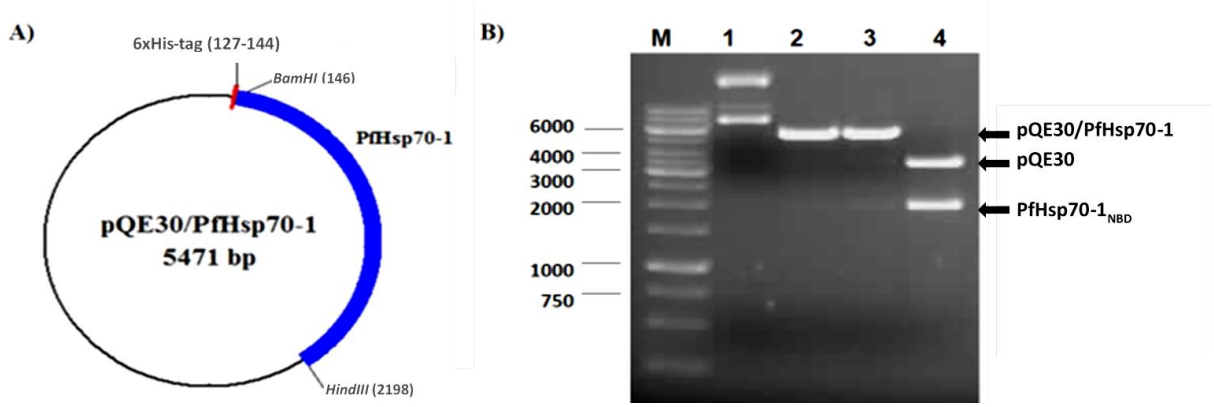


Figure 2.2 Restriction analysis of pQE30/PfHsp70-1 DNA plasmid
(A) Plasmid map of pQE30/PfHsp70-1 showing the *BamHI* and *HindIII* restriction sites. (B) Agarose gel electrophoresis of pQE30/PfHsp70-1: lane M, DNA molecular weight maker; lane 1, undigested pQE30/PfHsp70-1 plasmid; lane 2, pQE30/PfHsp70-1 digested with *BamHI*; lane 3, pQE30/PfHsp70-1 digested with *HindIII*; lane 4, pQE30/PfHsp70-1 digested with *BamHI* and *HindIII*.

2.3.2 Confirmation of pQE30/PfHsp70-1_{NBD} plasmid

The introduction of the *SpeI* restriction site into the pQE30/PfHsp70-1 plasmid by site directed mutagenesis to yield pQE30/PfHsp70-1_{NBD} was confirmed by agarose gel electrophoresis (Figure 2.3 B). Restriction enzymes *BamHI* and *SpeI* were used for restriction analysis to confirm the integrity of the pQE30/PfHsp70-1_{NBD} (Figure 2.3 B). Digestion with either *BamHI* or *SpeI* resulted in the linearised plasmid of 5471 bp (Figure 2.3 B). The PfHsp70-1_{NBD} construct (1201 bp), was confirmed by digesting the plasmid with both *BamHI*

and *SpeI*, which resulted in the DNA fragments of 4270 bp and 1201 bp (Figure 2.3 B). The integrity of the construct and insertion of the stop codon was confirmed by DNA sequencing (data not shown).

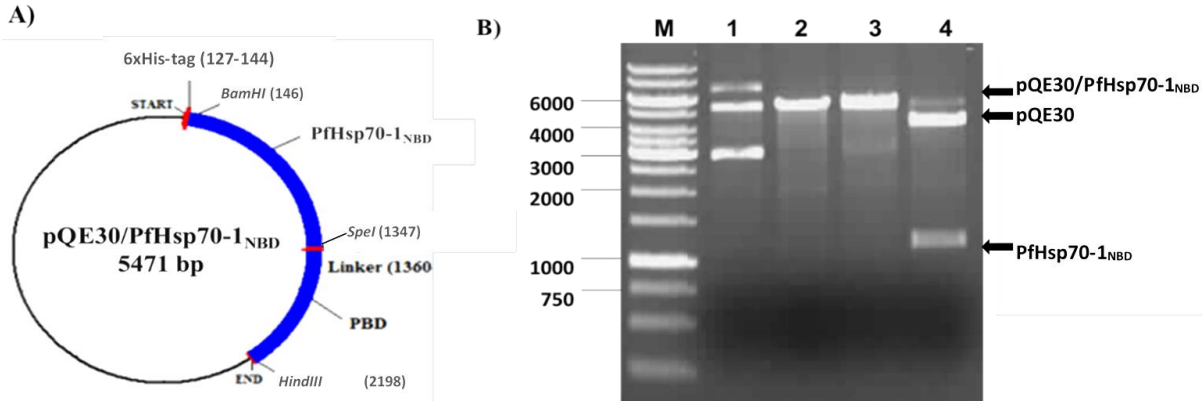


Figure 2.3 Restriction digest of pQE30/PfHsp70-1$_{NBD}$ construct
(A) Plasmid map of pQE30/PfHsp70-1$_{NBD}$ domain construct indicating the *BamHI* and *SpeI* restriction sites. (B) Agarose gel electrophoresis of pQE30/PfHsp70-1$_{NBD}$. The DNA samples were loaded onto the gel in the following order: lane M, molecular weight maker; lane 1, unrestricted pQE30/PfHsp70-1$_{NBD}$; lane 2, pQE30/PfHsp70-1$_{NBD}$ restricted with *BamHI*; lane 3, pQE30/PfHsp70-1$_{NBD}$ restricted with *Spe* I; lane 4, pQE30/PfHsp70-1$_{NBD}$ restricted with *BamHI* and *Spe* I.

2.3.3 Confirmation of pNI-His/PfHsp70-1 encoding PfHsp70-1

The *BamHI* and *HindIII* restriction sites on both pQE30/PfHsp70-1 and pNI-His facilitated the generation of the pNI-His/PfHsp70-1 plasmid encoding the PfHsp70-1 protein. The resultant plasmid, pNI-His/PfHsp70-1 was confirmed by both restriction analysis (Figure 2.4) and DNA sequencing (data not shown). Digestion with either *BamHI* or *HindIII* resulted in the migration of linearised pNI-His/PfHsp70-1 plasmid of 7066 bp (Figure 2.4 B). Insertion of the PfHsp70-1 coding sequence (2052 bp) was similarly achieved by subjecting the plasmid to digestion with *BamHI* and *HindIII*. Resulting in the 5014 bp and 2052 bp DNA fragments, corresponding to the pNI-His expression vector and PfHsp70-1 insert sizes, respectively (Figure 2.4 B).

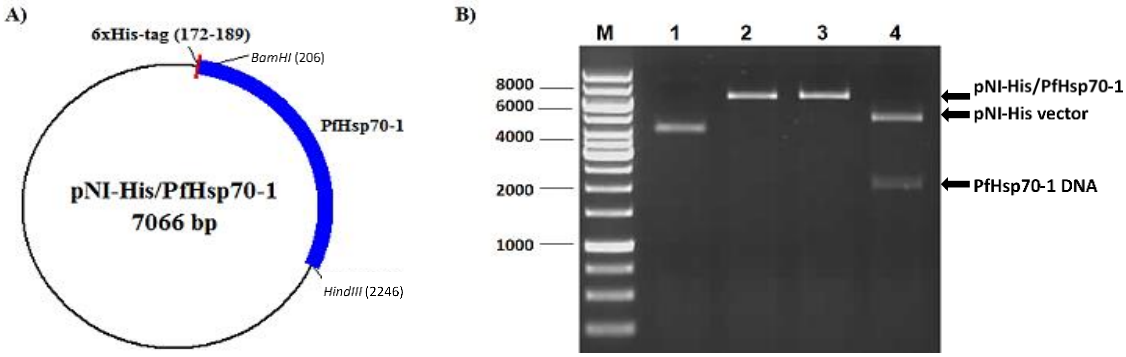


Figure 2.4 Restriction digest of pNI-His/PfHsp70-1 construct
(A) Plasmid map of of pNI-His/PfHsp70-1 construct indicating the *BamHI* and *Hind* I restriction sites. (B) Agarose gel electrophoresis of pNI-His/PfHsp70-1. The DNA samples were loaded onto the gel in the following order: M, DNA molecular weight maker; lane 1, represents unrestricted pNI-His/PfHsp70-1; lane 2, is the pNI-His/PfHsp70-1 restricted with *BamHI*; lane 3, represents pNI-His/PfHsp70-1 restricted with *HindIII*; lane 4, represents pNI-His/PfHsp70-1 restricted with *BamHI* and *HindIII*.

2.3.4 Confirmation of pKK223-2/PfLDH plasmid encoding PfLDH

Restriction digest of pKK223-2/PfLDH was carried out to verify the integrity of the construct (Figure 2.5; Krause, 2011). Digestion of pKK223-2/PfLDH using either *PstI* or *EcoRI* resulted in the linearised pKK223-2/PfLDH plasmid fragment of 5521 bp (Figure 2.5 B). The insertion of PfLDH encoding sequence was confirmed by the digesting pKK223-2/PfLDH plasmid with either *PstI* or *Eco* RI. Resulting in the 5014 bp and 2052 bp DNA fragments, corresponding to the pNI-His expression vector and PfHsp70-1 insert sizes, respectively (Figure 2.5 B). DNA sequencing (data not shown) analysis was further used to verify the integrity of the plasmid.

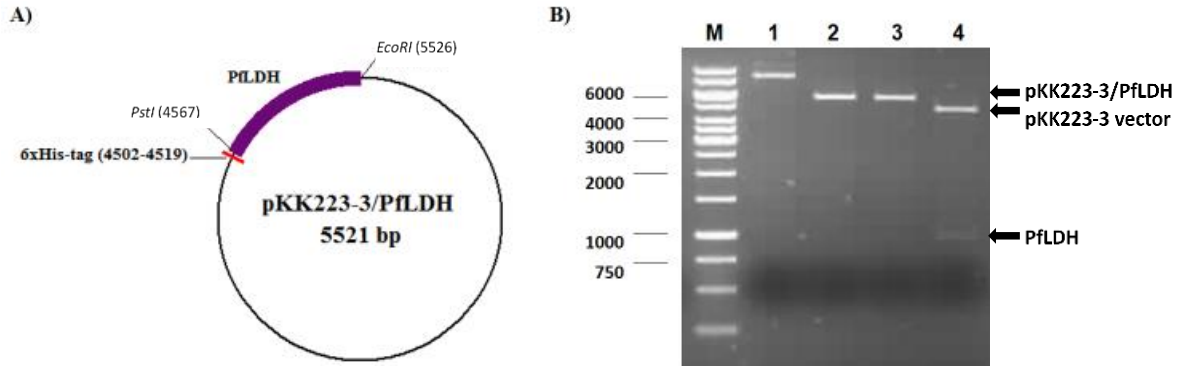


Figure 2.5 Restriction digest of pKK223-3/PfLDH construct
(A) Plasmid map of of pKK223-3/PfHLDH construct indicating the *BamHI* and *SpeI* restriction sites. (B) Agarose gel electrophoresis of pKK223-3/PfLDH. The DNA samples were loaded onto the gel in the following order: M, molecular weight maker; lane 1, unrestricted pKK223-3/PfHLDH; lane 2,

pKK223-3/PfHLDH restricted with *Pst* I; lane 3, pKK223-3/PfHLDH restricted with *Eco* RI; lane 4, pKK223-3/PfHLDH with *PstI* and *Eco* RI.

2.3.5 Expression PfHsp70-1 in *Brevibacillus choshinensis*

Recombinant PfHsp70-1 protein was successfully expressed in *B. choshinensis* cells (Figure 2.6). PfHsp70-1 (74 KDa) production was analysed by SDS-PAGE and confirmed by Western blot analysis using α-PfHsp70-1 (Figure 2.6). PfHsp70-1 was successfully detected from culture media without cells, and also inside the expression cells. Indicating that PfHsp70-1 was expressed inside the *B. choshinensis* and was also secreted to the extracellular culture media (Figure 2.6). Western analysis indicated that PfHsp70-1 in the intracellular fragment was detected with various species at approximately 35 kDa, 74 KDa and 100 kDa, which were likely to be protein breakdown products and possible dimerisation products (Figure 2.6). Whereas, in the extracellular medium PfHsp70-1was detected by Western analysis as single species approximately 74 kDa (Figure 2.6). In this study PfHsp70-1 expressed into the extracellular medium could not be purified by affinity chromatography. PfHsp70-1 secreted into the extracellular medium may have been digested by proteases in the media.

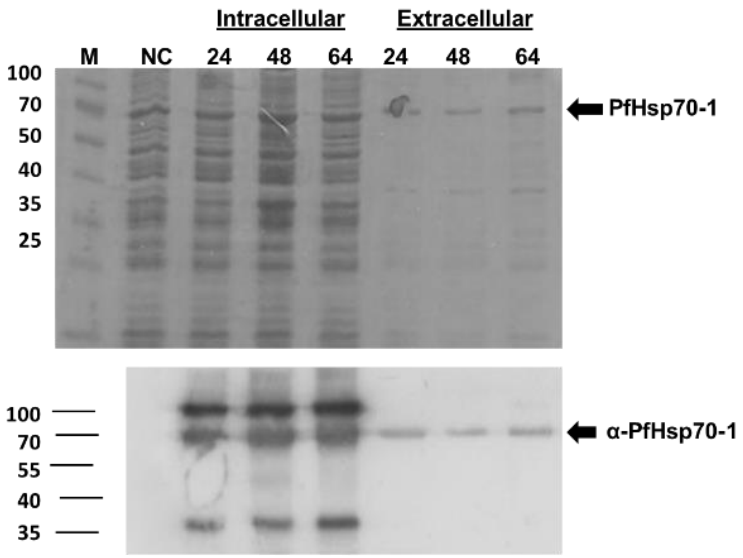


Figure 2.6 Intracellular and extracellular expression of recombinant PfHsp70-1
PfHsp70-1 was over-expressed in *B. choshinensis* proteins were successfully purified by nickel-affinity chromatography. PfHsp70-1 expression and purification were analysed by 12.5% SDS-PAGE (upper panel) and confirmed by Western blot (lower panel) using α-PfHsp70-1 to confirm the presence of PfHsp70-1. Lane NC, untransformed cells; lane 24, 48 and 64 are samples collected 24 hours, 48 hours and 64 hours post-protein induction. Lane M, protein molecular weight marker.

2.3.6 Purification of PfHsp70-1 from *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis.*

The production of PfHsp70-1 was visualized by SDS-PAGE analysis and confirmed by Western blot analysis (Figure 2.7). Recombinant PfHsp70-1 protein was expressed in *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* cells as a protein species of approximately 74 kDa. No PfHsp70-1 was detected in the untransformed *E. coli* ClearColi BL21 control lane (Figure 2.7 A, D, G; lane NC). PfHsp70-1 protein was successfully expressed using *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* in response to induction (Figure 2.7 A, D, G). Western analysis on transformed *B. choshinensis* cells using α-PfHsp70-1 indicated the presence of PfHsp70-1 in the un-induced samples (Figure 2.7 D; lane 0). This indicates "leaky" protein expression observed in the un-induced samples, when using *B. choshinensis.* PfHsp70-1 cultures were harvested overnight (O/N) upon reaching optimal expression.

Although the use of the His_6-tag's in protein purification has been reported to reduce the ATPase activity of the purified products, the affinity tag is vital for reducing non-specific endogenous protein co-purifications (Terpe, 2002). PfHsp70-1 produced from the three bacterial strains was expressed attached to a specific N-terminal His_6-tag and purified using nickel affinity chromatography, in order to eliminate endogenous Hsp70 co-purifying with the protein of interest (Figure 2.7). PfHsp70-1 authenticity was confirmed by Western blot analysis using α-PfHsp70-1 and α-His antibodies (Figure 2.7). PfHsp70-1 expressed in *E. coli* XL1 Blue and *E. coli* ClearColi BL21 cells was successfully purified using affinity chromatography, minimal recombinant PfHsp70-1 protein was lost through the wash step

(Figure 2.7 A and D; lane W) resulting in most of the PfHsp70-1 protein being eluted (Figure 2.7 A and E; lane E1 and E2).

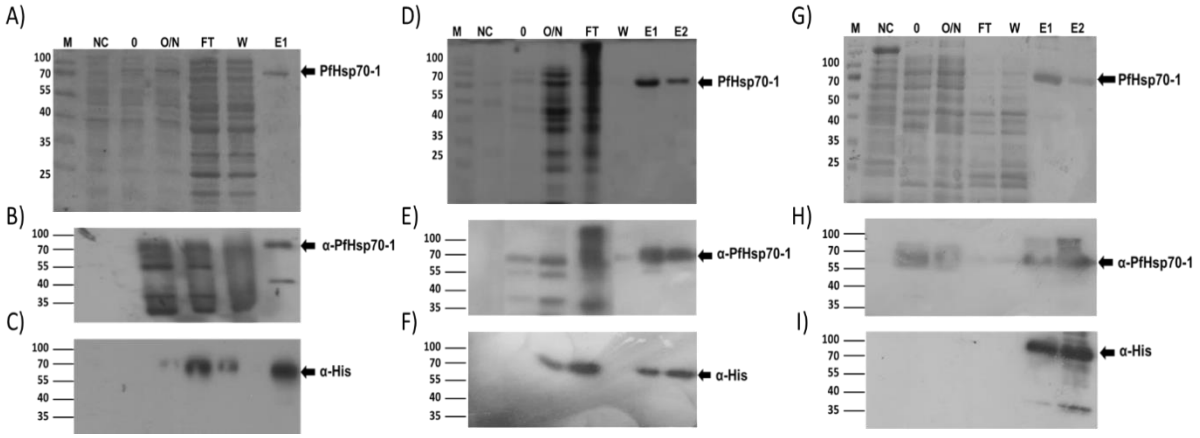


Figure 2.7 Expression and purification of recombinant PfHsp70-1 in the absence and presence of LPS

PfHsp70-1 (74 kDa) was over-expressed in E. coli XL1 Blue (A), E. coli ClearColi BL21 (D) and B. choshinensis (G), the proteins were successfully purified by nickel-affinity chromatography. PfHsp70-1 expression and purification as analysed using 12.5% SDS-PAGE (upper panel). Lane NC, are untransformed cells; lane 0, pre-induction; and lane O/N represent overnight post-protein induction culture samples. Lane FT, flow-through samples, lane W, wash buffer samples and lane, E1 and E2, represent the eluted protein samples. M, protein molecular weight marker. The α-PfHsp70-1 antibody (B, E and H) and α-His antibody (C, F and I) were used to confirm the presence of the purified proteins.

Recombinant PfHsp70-1 expressed using *B. choshinensis* cells was also purified using nickel affinity chromatography (Figure 2.7 G; lane E1 and E2). Minute amounts of PfHsp70-1 were lost through the wash step (Figure 2.7 G; lane W), resulting in most of the protein being recovered in the elution samples (Figure 2.7 G; lanes E1 and E2). The PfHsp70-1 species detected during protein expression at approximately 35 kDa and 100 kDa did not co-purify with PfHsp70-1 protein, suggesting that they had no polyhistidine tag (section 2.3.6; Figure 2.7 G; lane E1 and E2). It is likely that the species of lower molecular weight detected by Western analysis using α-PfHsp70-1 and α-His were an N-terminus truncated version of PfHsp70-1 or perhaps the degradation products that as a results of sample preparation and handling (Figure 2.7 H and I; lane E1 and E2). Purifications of PfHsp70-1 across all three

expression systems typically resulted in a final protein yield of approximately 400 mg/L; which was sufficient for subsequent *in vitro* assays.

2.3.7 Expression and purification of PfHsp70-1_{NBD}

To further identify the interaction between the LPS and PfHsp70-1, the truncated form of PfHsp70-1 (PfHsp70-1_{NBD}) was over-expression and purification in *E. coli* ClearColi BL21 (Appendix B. 7; Figure B. 7) and *E. coli* XL1 Blue cells (Figure 2.8). PfHsp70-1_{NBD} lacks the C-terminal that is believed crucial for binding to LPS (Habich *et al*., 2005). The production of PfHsp70-1_{NBD} at 45KDa was confirmed by SDS-PAGE analysis (Figure 2.8). Before IPTG induction, no protein band was observed at the expected mobility range (Figure 2.8; lane 0). However, upon adding IPTG, the cells were able to express PfHsp70-1_{NBD} after 1 hour induction. PfHsp70-1_{NBD} cultures were harvested after reaching optimal expression, 5 hours post-IPTG induction (Figure 2.8; lanes 5). PfHsp70-1_{NBD} was purified natively using affinity chromatography. PfHsp70-1_{NBD} production was analysed by SDS-PAGE and confirmed by Western blot analysis using polyclonal α-PfHsp70-1 antibody (Figure 2.8). Purifications of PfHsp70-1_{NBD} resulted in a final protein yield of approximately 600 mg/L; sufficient for subsequent *in vitro* assays.

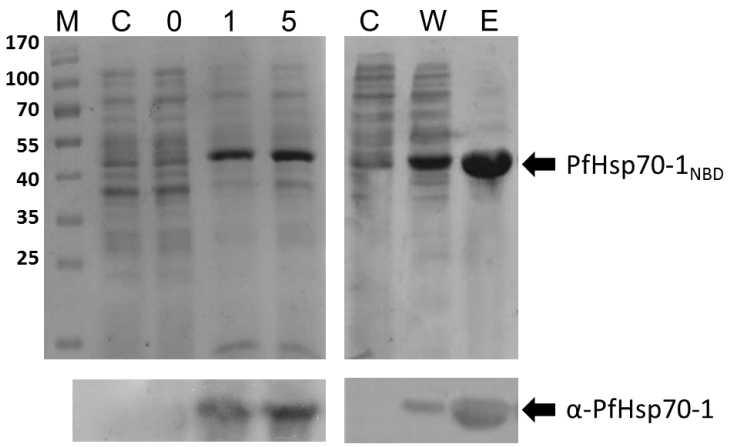


Figure 2.8 Successful expression and purification of PfHsp70-1_{NBD}
PfHsp70-1_{NBD} (45 kDa) was over-expressed in *E. coli* ClearColi BL21 cells. The proteins were successfully purified by nickel-affinity chromatography. PfHsp70-1_{NBD} expression and purification

were analysed by 12.5% SDS-PAGE (upper panel). Lane C, untransformed cells; lane 0, pre-induction; lane 1, 1 hour post-IPTG induction and lane 5, 5 hours post-IPTG induction. M, molecular weight marker of recombinant proteins. Lane W, wash buffer samples and lane E, the eluted protein samples Western blot analysis α-PfHsp70-1 polyclonal antibody (1: 2000) was used to confirm the presence of the PfHsp70-1_{NBD} (lower panel).

2.3.8 Expression and purification of PfLDH

PfLDH is a malarial protein that has no known chaperone activity. Thus, it is an ideal negative control to evaluate the capability of expressing LPS-free proteins using *E. coli* ClearColi BL21. Thus, recombinant PfLDH was over-expressed at 36 KDa in *E. coli* ClearColi BL21 and purified by affinity chromatography. PfLDH was eluted with at 36 KDa, with secondary dimer species around 70 kDa. Protein expression and purification was analysed by SDS-PAGE and confirmed by Western blot analysis using polyclonal α-His antibody (Figure 2.9). The protein was extensively dialysed and stored for later use in cytokine expression studies in Chapter 3. Since *B. choshinensis* cells do not harbour LPS molecules, the PfHsp70-1_{NBD} encoding plasmid in *B. choshinensis* was not required for subsequent analysis in this report hence it was not constructed.

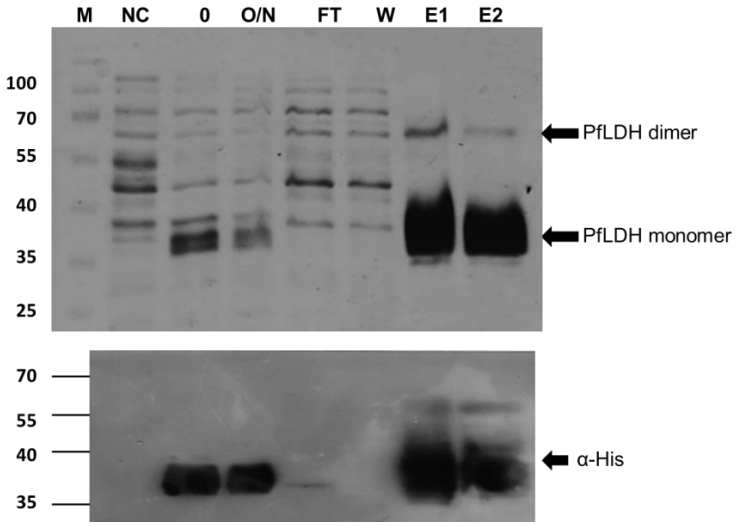


Figure 2.9 Expression and purification of recombinant PfLDH
PfLDH (35 kDa) was over-expressed in *E. coli* ClearColi BL21 cells. PfLDH expression and purification was analysed using SDS-PAGE (upper panel) and α-His antibody was used to confirm the presence of the polyhistidine tag. Lane M, molecular weight protein marker. Lane NC, untransformed cells; lane 0, pre-induction; lane O/N are overnight post-protein auto-induction culture samples. Lane FT, flow through sample; lane W, protein purification wash buffer sample and lane, E1 and E2, eluted protein samples.

2.3.9 Addition of ATP reduces LPS co-purification on PfHsp70-1

Hsp70s possess the dual signalling between the NBD and peptide binding domain (PBD) (section 1.4.2). The allosteric function promotes release of substrates when ATP is bound in the NBD motif (Liberek *et al*. 1991; Suh *et al*., 1999). The stripping of LPS from PfHsp70-1 presents an effective purification technique for reduced LPS contamination.The EndoLISA kit (Hyglos GmbH, Bernried, Germany) was used to measure the amount of endotoxin in the final protein concentrations purified from *E. coli* XL1 Blue cell. To remove the LPS contaminants, two batches of PfHsp70-1 purified from *E. coli* XL1 Blue cells (ATP supplemented and non-ATP supplemented) were allowed to flow through an Endotrap Red coloumn (Hyglos GmbH, Bernried, Germany). The addition of ATP to purified PfHsp70-1 may have promoted the release of the bound peptide substrates by the chaperone as a decrease in sample endotoxin contamination was noted (1.88 ng/ml LPS) as compared to the non-ATP spiked (13.63 ng/ml LPS) for PfHsp70-1 protein at the same concentration (Figure 2.10).

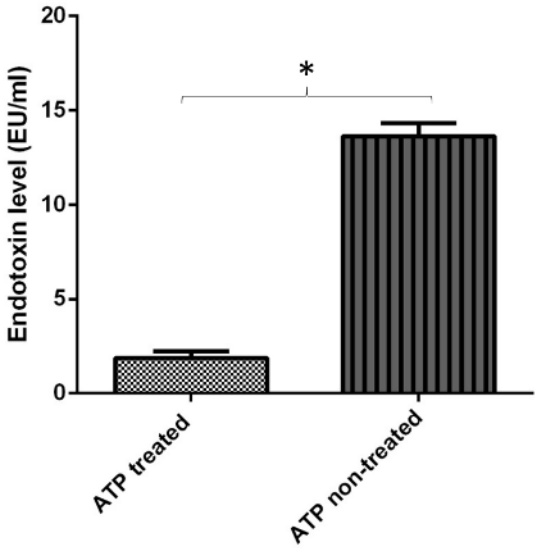


Figure 2.10 The addition of ATP to PfHsp70-1 during purification reduces endotoxin contamination
Analysis of LPS content of PfHsp70-1 purified with and without addition of ATP. The addition of ATP to PfHsp70-1 produced using *E. coli* XL1 Blue exhibits reduced endotoxin contamination. Student t-test was used calculate statistical differences (*$P < 0.001$).

2.3.10 Purification of recombinant PfHsp70-1 expressed in *E. coli* ClearColi BL21 and *B. choshinensis*

The production of recombinant proteins without LPS contamination has proven to be problematic yet very essential in immune studies (Gao and Tsan, 2003). Genetically modified *E. coli* ClearColi BL21 and *B. choshinensis* cells are believed to express recombinant proteins excluding LPS contamination. To confirm the ability to produce proteins without endotoxin; equal amounts of PfHsp70-1 expressed using *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* were analysed for endotoxin contamination using the LAL assay. As expected, increasing concentration of PfHsp70-1 purified from *E. coli* XL1 Blue, were associated with proportional levels of LPS contamination (Figure 2.11). Intrestingly, LPS detected from PfHsp70-1 purified in *E. coli* ClearColi BL21 and *B. choshinensis* expression systems were significantly low ($P < 0.001$), compared to LPS detected on PfHsp70-1 produced in *E. coli* XL1 Blue expression host (Figure 2.11).

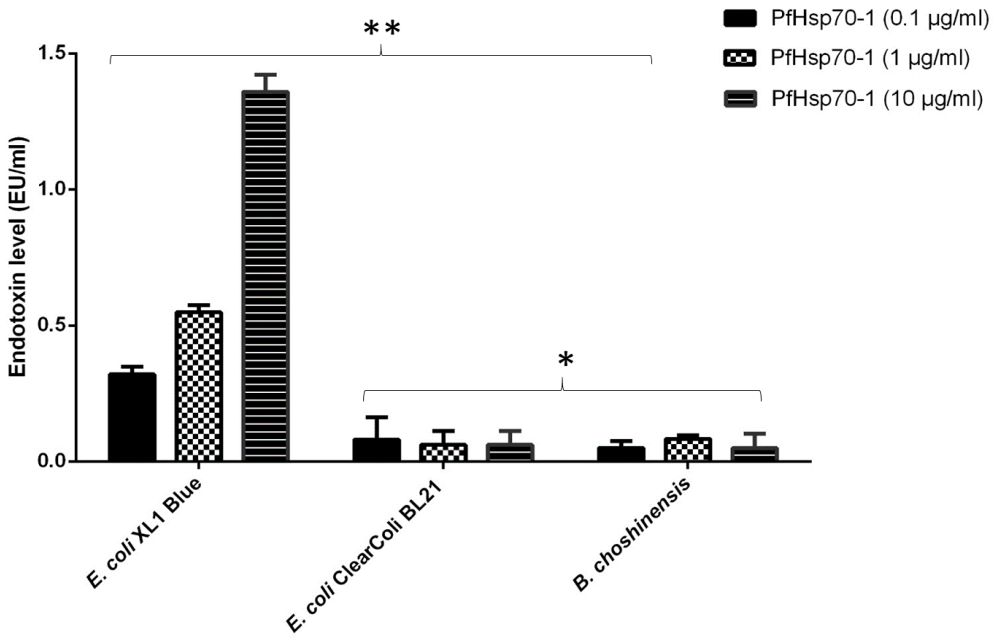


Figure 2.11 Analysis of PfHsp70-1 endotoxin contamination
LPS contamination was measured using the LAL assay from PfHsp70-1 produced using *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* expression hosts. The mean endotoxin contamination are represented by the vertical bar graphs and the respective standard errors of the mean are indicated for each expression system. Two-way ANOVA was used to calculate the statistical differences (*$P \geq 0.05$, **$P < 0.001$).

Since *B. choshinensis* cell do not produce LPS molecules the observed LPS-contamination levels detected on PfHsp70-1 produced in *E. coli* ClearColi BL21 and *B. choshinensis* may have been as a result of experimental errors or LAL assay inability to distinguish between LPS variants. Nonetherless, these results appear to indicate that recombinant proteins can successfully be produced in *E. coli* ClearColi BL21 and *B. choshinensis* cells without LPS contamination.

2.3.11 PfHsp70-1 produced in the absence LPS is biologically active

Proteins show sensitivity to various handling conditions These modifications can alter the protein structure leading to protein aggregation and reduced activity (Lu *et al*., 2001). The production of properly folded heterologous proteins directly confers to their structural stability and biological activity (Opekarova and Tanner, 2003; Tate *et al.,* 2003). The study, therefore, utilised the ATPase assay to evaluate the biological activity of PfHsp70-1 produced in *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis*. No activity was observed in the negative control, indicating that the observed readings were not due to spontaneous hydrolysis of ATP (data not shown). The basal ATP hydrolysis activity of PfHsp70-1 purified from *E. coli* XL1 Blue and *E. coli* ClearColi BL21 were calculated as 23.6 ± 1.5 nmoles Pi/min/mg and 21.6 ± 1.8 nmoles Pi/min/mg, respectively (Figure 2.12). The reported activity is within range of previously reported data (Matambo *et al*., 2004; Shonhai, 2007). The basal activity of PfHsp70-1 purified from *B. choshinensis* was found to be 36.8 ± 1.2 nmoles Pi/min/mg, significantly higher ($P < 0.001$) than that of PfHsp70-1 purified from *E. coli* XL1 Blue and *E. coli* ClearColi BL21 (Figure 2.12). These results indicate that biologically active recombinant PfHsp70-1 proteins can be produced using *E. coli* ClearColi BL21 and *B. choshinensis* in the absence of LPS contamination.

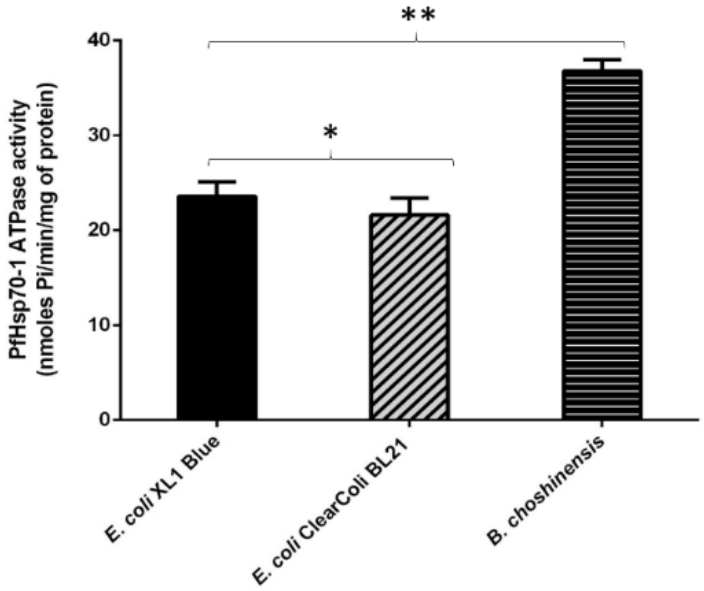


Figure 2.12 Analysis of the ATPase activity of PfHsp70-1 purified in the absence and presence LPS

PfHsp70-1 purified in the absence of LPS using *E. coli* ClearColi BL21 and *B. choshinensis* had its basal ATPase activity analysed in comparison to that of PfHsp70-1 purified from *E. coli* XL1 Blue cells. EC and BC represent *E. coli* and *B. choshinensis* respectively. One-way ANOVA was used to show statistical differences (*$P \geq 0.05$, **$P < 0.001$).

2.4 Discussion

Heterologous protein expression of malarial proteins has largely been conducted in *E. coli* (Terpe, 2005). Studies utilising Gram-positive bacteria such as *B. subtilis*, in protein expression studies, offer advantage as the expressed protein is secreted directly into the culture media thus circumventing high intracellular expression limitations, such as formation of inclusion bodies (Harwood, 1992). PfHsp70-1 produced in *E. coli* XL1 Blue was comparable to PfHsp70-1 produced in *E. coli* ClearColi BL21 and *B. choshinensis*. The reliability of *E. coli* ClearColi BL21 as an efficient expression host was further demonstrated by its ability to successfully express PfLDH and PfHsp70-1_{NBD} in soluble form. This report is one of the few studies, if not the first, to report the successful expression of malarial PfHsp70-1 with minimal contamination. Prugnolle and co-workers (2008) reported that *Plasmodium* genes with high GC content are more likely to be expressed ubiquitously; whereas low GC-rich genes are likely to be lowly expressed. Since *B. choshinensis* cells are GC-rich (Takagi et al, 1993), the *Brevibacillus* expression system may potentially be used to produce malaria proteins that have previously failed to express in *E. coli* systems, especially those with high GC content. Thus, this expression system may contribute to future efforts to study the structure and function of *P. falciparum* proteins.

The basal ATP hydrolysis activity of PfHsp70-1 purified from *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* cells were compared in order to analyse the biological activity of proteins produced from the three expression hosts. Basal ATP hydrolysis activities of PfHsp70-1 purified from *E. coli* XL1 Blue and *E. coli* ClearColi BL21 were comparable with previously reported data (Matambo *et al*., 2004; Shonhai *et al*., 2007). However, in this study the basal activity of PfHsp70-1 purified from *B. choshinensis* was significantly higher than that purified from *E. coli* XL1 Blue and *E. coli* ClearColi cells. Gram-positive protein

expression systems are believed to possess better protein folding and reducing conditions as compared to *E. coli* expression system (Westers *et al*., 2004; Talarico *et al*., 2005; D'Urzo *et al*., 2013). Talarico and colleagues (2005) reported a high enzymatic protein activity of proteins expressed in *Bacillus megaterium* as compared to those expressed in *E. coli*. They argued that better protein folding conditions and a better codon usage in *B. megaterium* expression systems was responsible for better yield (Talarico *et al*., 2005). These data indicates that recombinant proteins expressed in *B. choshinensis* are better folded as compared to protein expressed in *E. coli* XL1 Blue and *E. coli* ClearColi BL21. Hence, the elevated biological activity displayed by PfHsp70-1 protein expressed in *B. choshinensis*.

PfHsp70-1 has previously been shown to have a reduced affinity for MDH, in the presence of ATP (Shonhai *et al*., 2008). The addition of ATP triggered a conformational change that resulted in the release of bound peptide from PfHsp70-1 (Shonhai *et al*., 2008). HSPA1A and Hsc70 have been shown to directly interact with lipids and that the addition of ADP, inhibits Hsp70-induced liposome aggregation (Arsipe *et al*., 2002; McCallister and Nikolaidis, 2014). Indicating that Hsp70 may potentially bind and interact with lipid-based molecules. Habich and co-workers (2005) showed that Hsp60 proteins directly bind to LPS through their C-terminal, possibly through their chaperone role. Interestingly, murine Hsp70s treated with ATP have previously been demonstrated to release immunogenic peptides bound to the Hsp70, whereas ADP-treatment did not (Wang *et al*., 2006). Similarly, in this study, the addition of ATP during purification of PfHsp70-1 expressed from *E. coli* XL1 Blue resulted in the reduction of LPS contamination associated with the protein (1.88 ± 0.5 ng/ml LPS) as compared to protein purified in the absence of ATP (13.63 ± 1.52 ng/ml LPS). The addition of ATP to PfHsp70-1 may have led to the release of bound peptide substrates, hence the reduction of LPS associated with PfHsp70-1. Moreover, purified PfHsp70-1_{NBD} contained

lower LPS contamination compared to full length PfHsp70-1 (data not shown), possibly due to its lack of the PBD. These results suggest that LPS predominantly binds to PfHsp70-1 through its C-terminal. These findings appear to suggest that PfHsps70-1 may bind to LPS through its C-terminal as previously reported in experiments using Hsp60 by Habich and co-workers (2005). Although the LAL assay has widely been used in the detection of endotoxins, the assay has been reported to lack the ability to differentiate between LPS variants (Sushruta and Anubha, 2011). Furthermore, this is largely due to the fact that the LAL assay recognises some conserved residues on the backbone LPS and not the acylation pattern of LPS for endotoxin detection (Mamat *et al*., 2013). The observed traces of LPS detected from PfHsp70-1 produced in *E. coli* ClearColi BL21 and *B. choshinensis* levels were most likely due to the nonspecific nature of the LAL assay. Hence, Mamat and colleagues (2013) recommended endotoxin detection assays using immune cells as they are more specific and can detect the presence of endotoxin at levels below the LAL assay thresholds. Hence, Chapter 3 investigated the immunomodulatory function of recombinant PfHsp70-1 using HEK293 TLR4 transfected cells, wild-type murine macrophages and PMN cells.

CHAPTER 3

EXPLORING THE IMMUNOMODULATORY FUNCTION OF RECOMBINANT *Plasmodium falciparum* HSP70-1

3.1. Introduction

The innate immune cells, such as neutrophils (PMN), dendrite cells (DCs) and macrophages play a pivotal role in coordinating the host immune system. When triggered by foreign agents, such as bacteria or bacterial components these cells produce pro-inflammatory cytokines, such as interleukins (IL) (Kumar and Sharma, 2010). The induced cytokines are crucial for the organisation of the innate and adaptive immune system (Seymour and Gemmell, 2001; Kumar and Sharma, 2010). Toll-like receptors (TLRs) are highly preserved pattern recognition receptors found in immune cells (section 1.8; Lundberg *et al*., 2007). TLRs have been shown to differentiate between self or non-self molecules. TLR transfected recombinant Human Embryonic Kidney 293 cells (HEK293) cells have been used to investigate TLR-specific ligands (Birchler *et al*., 2001; Heine *et al*., 2003; 2011). HEK293 cell lines were initially transformed by Frank Graham on his 293rd experiment (Graham *et al*., 1977**;** Huang *et al*., 2009). HEK293 cells are human embryonic kidney cells that can be efficiently transfected to enable the production or expression of specific receptors or protein of interest. Thus enabling characterisation of microbial ligands at molecular level (Huang *et al*., 2009).

TLRs are responsible for recognising conserved pathogen-associated molecular patterns (PAMPs) (section 1.8). TLR stimulations are known to trigger intracellular signalling pathways resulting in the synthesis and expression of various cytokines (Takeuchi and Akira, 2010). In addition to PAMPs, TLRs can also be stimulated by molecules derived from host tissues or cells. Extracellular matrix components such as such as fibronectin, biglycan and fibrinogen have been shown to be activate the immune system (Jiang *et al*., 2005; Schaefer *et al*., 2005; Taylor *et al.,* 2007). Endogenous Hsps have also been shown to stimulate TLR 2 and TLR 4, thus are thought to function as chaperokines (section 1.8; Asea *et al*., 2002;

Chase *et al*., 2007; Wheeler *et al*., 2009). Hsp70 proteins are conserved molecules that constitute a major part of the cell's protein folding machinery (section 1.4.2). Parasite Hsp70 proteins have previously been documented as potent immune activators (Mun *et al*., 2000). For example, TgHsp70 has been shown to modulate host immunity by inducing the production of immune factors, such as cytokines (Hwang *et al*., 2010). However, other studies have suggested that Hsp-induced signalling may be as the results of endotoxin contamination produced along with these recombinant proteins (Bausinger *et al*., 2002; Tsan and Gao, 2009). Since, Hsp60, Hsp70 and Hsp90 have been shown to bind bacterial LPS (endotoxin) (Habich *et al*., 2005; Ambade *et al*., 2012). Poulaki and colleagues (2012) showed that endotoxin-induced inflammation is reduced by the inhibition of Hsp90. Hsps are believed to bind LPS through their chaperone function. Thus, inducing the production of pro-infammatory cytokines (Ambade *et al*., 2012).

A number of techniques have been employed to neutralise the adverse effects of LPS co-purified with recombinant proteins. Since, LPS is not sensitive to heat (section 2.1), heat denaturation has been used to evaluate the immunostimulatory effect of Hsps on macrophages. Wang and colleagues (2006) demonstrated that Hsp70 purified in the absence of LPS successfully managed to stimulate macrophages cells. Cationic antibiotics such as polymyxin-B (section 4.1) have previously been used to neutralise the immunostimulatory effects of LPS (Bausinger *et al*., 2002; Wallin *et al*., 2002). Controversially, other studies have proposed heat denaturation and polymyxin-B treatment inadequately suppresses the immunostimulatory role of LPS on DCs (Tynan *et al*., 2012). Hence, it is crucial that studies investigating the immunostimulatory role of Hsps use protein produced in the absence of LPS contamination to elucidate this confounding data. Thus, the study sought to investigate the role of PfHsp70-1 produced without LPS contamination in order to resolve the controversy

surrounding Hsps and their chaperokine role on immune cells. In this study, the immunomodulatory role of recombinant PfHsp70-1 was investigated produced in *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* expression hosts.

The objectives of the study were to:

1) Determine the expression of cytokines (IL6 and IL8) by murine DCs and HEK transfected TLR4 cells exposed to recombinant PfHsp70-1.
2) Explore the effect of polymyxin-B on IL6 and IL12 expression on Macrophages treated with recombinant PfHsp70-1 produced in *E. coli* XL1 Blue cells.
3) Conduct flow cytometry analysis on PMN treated with PfHsp70-$1_{NBD.}$

3.2. Experimental procedures

3.2.1 Materials

Endotoxin free water, sodium azide, wild type murine dendrite cells of BALB/c mouse origin were donated by Dr. Gabi Koellisch (Phillip University Marburg, Germany). TLR 4 and TLR2 transfected HEK293 macrophage cells, were graciously provided by Prof. Dr. Holger Heine (Borstel Research Centre, Germany). Human PMN were isolated using the histopaque gradient method (Appendix B5) from healthy adult blood volunteers. Peripheral blood was collected by veripuncture, informed consent was obtained from the volunteers.

3.2.2 Cell cultures and stimulation assays

Most investigators focusing on the chaperokine functions of Hsps use recombinant proteins produced from *E. coli.* LPS is immunogenic and is recognised receptors, such as toll-like receptor 4 (TLR4), on innate immune cells (monocytes, macrophages, neutrophils (PMN) and Macrophages (Fiske *et al*., 2001). Cytokine induction assays were conducted to investigate the immunomodulatory role of PfHsp70-1 purified from *E. coli* XL1 Blue, *E. coli* ClearColi BL21 and *B. choshinensis* expression hosts. Murine macrophage cells of BALB/c mouse origin were maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and 2% AS-serum at 5% CO_2 and 37°C. Cell stimulation assays started immediately after cell isolation or from cells retrieved from storage at -80°C and cultured at 3 X 10^6 cells/ml. For the induction of cytokine release, cells were cultured for 24 hrs in the presence of various concentrations (0, 100 and 500 ng/ml) of PfHsp70-1 purified from *E. coli* XL1 Blue and *E. coli* ClearColi BL21expression hosts. Cells were also co-cultured with 10 ng/ml of LPS, a positive immune cell activator. After incubation the cell culture supernatants were collected and analysed by IL6 (BD Bioscience, San Jose, USA) and mIL12p40 (BD

Bioscience, San Jose, USA) expression following manufactures instruction. To further test the reliability of proteins purified from *E. coli* ClearColi BL21 cells. Purified forms of PfLDH and PfHsp70-1_{NBD} expressed using *E. coli* ClearColi BL21 cells were also seeded with murine macrophage cells and observed for IL6 and mIL12p40 cytokine expression.

TLR4 and TLR2 transfected HEK293 macrophage cells, were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with U/ml penicillin, 100 µg/ml streptomycin and FBS (10%). For the induction of IL8 production, transfected HEK293 cells were cultured for 24 hrs in the presence of various concentrations (0, 0.25 and 2.5 µg/ml) of PfHsp70-1 purified from *E. coli* XL1 Blue and *B. choshinensis* respectively. Cells were also cultured with LPS a known TLR4 macrophages activator. After incubation the cell-free supernatant were collected and analysed by IL8 (Life Technologies GmBH, Germany) production following manufactures protocol.

PMNs were cultured immediately after isolation at 37°C (5% CO_2) incubator, using complete medium (RPMI 1640 medium supplemented, 10 mM HEPES, 10% heat inactivated fetal bovine serum (all from Sigma-Aldrich, Steinheim, Germany), 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all purchased from Biochrom, Berlin, Germany). To investigate the relationship between LPS and *E. coli* XL1 Blue recombinant protein's ability to stimulate cytokine induction. Therefore, various concentrations (0.01, 0.04, 0.2 and 1 ng/ml) of LPS were boiled at 100°C for 10 minutes and later left to cool. Boiled and non-boiled LPS were then seeded with PMNs with 5X 10^6 cells/ml at the indicated concentrations and cultured overnight at 37°C (5% CO_2). Non boiled LPS at similar concentrations were also cultured with PMN under the same conditions. The effect of heating recombinant proteins ability to induce on immune-cell cytokine production, a fraction PfHsp70-1_{NBD}

protein was boiled at 100°C for 10 minutes and then left to cool. Boiled and non-boiled PfHsp70-1_{NBD} at various concentrations (0, 100, 250 ng/ml), were cultured with the isolated PMN (5 X 10^6 cells/ml) overnight. PMN purity was assessed by size and granularity using a FACS Calibur flow cytometer and the CellQuest Prosoftware (BD Biosciences, San Diego, USA). (BD Bioscience). After incubation the supernatant was collected and analysed for IL8 release. (Life Technologies GmBH, Germany) production following manufactures protocol.

3.2.3 Cytokine expression analysis

Cytokine levels in culture supernatants were measured using cytokine-specific ELISA assays IL8, IL6 and IL12 (All R&D Systems) according to the manufacturer's guidelines (BioRad, Hercules, CA). Murine Macrophages and were grown at 37°C (5% CO_2) in cell culture medium in the presence of PfHsp70-1 at various concentrations (0, 100 and 500 ng/ml) from the three bacterial expression systems, cytokine (IL6 and IL12) content were quantified by ELISA to monitor cell activation. HEK293 TLR4 transfected cells were similarly cultured at 37°C (5% CO_2) with PfHsp70-1 (0, 0.25 and 2.5 µg/ml) purified from the three bacterial expression hosts. IL8 content was quantified using culture supernatants by ELISA (Biosource, Solingen, Germany).

3.2.4 Flow cytometry analysis

Innate cells such as, PMN cell rapidly shed CD62-L or L-selectin receptors from the surface of the outer cell membrane, when activated by the presence LPS. Cell activation was measured by monitoring CD62-L shedding using fluorescein isothiocyanate (FITC)-conjugated antibodies (BD Biosciences, San Jose, CA). For live cell staining, PMNs cells were stained with CD62L-FITC antibodies following the standard protocol (Appendix A.6). Briefly, FACS buffer (PBS containing 1% FBS, 1% BSA, 0.01% sodium azide) and CD62-L

antibodies were added directly to 5 x 10^5 cells followed by 30 min incubation at 4°C in the dark, the cells were washed twice with FACS buffer. The cells were re-suspended cells in FACS buffer with 1% Paraformaldehyde (Sigma-Aldrich, Saint Louis, MO) to a final concentration 5 µg/ml before FACS analysis. All data was assessed using the flow cytometry (BD FACS Calibur) and the CellQuest Prosoftware (BD Biosciences, San Diego, USA).

3.3. Results

3.3.1 Assessment of immune cell activation function of PfHsp70-1

LPS are pyrogenic molecules that bind to recombinant proteins when purifying proteins produced using traditional *E. coli* expression systems. This part of the study focussed on investigating the immunostimulatory role of PfHsp70-1. Wild-type murine Macrophages cultured in media alone did not induce cytokine expression (Figure 3.1). Macrophages cultured in the presence of PfLDH did not induce cytokine expression (Appendix B; Figure B5). Whereas, cells cultured in the presence of LPS were successfully stimulated to induced IL6 expression (Figure 3.1). These results indicated that the cytokine expression was not induced by external contamination. Interestingly, murine Macrophages cultured with PfHsp70-1 purified from *E. coli* XL1 Blue cells were significantly stimulated to produce IL6. Whereas, Macrophages treated with the same concentration of PfHsp70-1 prepared in *E. coli* ClearColi BL21 expression host was significantly low ($P \leq 0.00143$) (Figure 3.1).

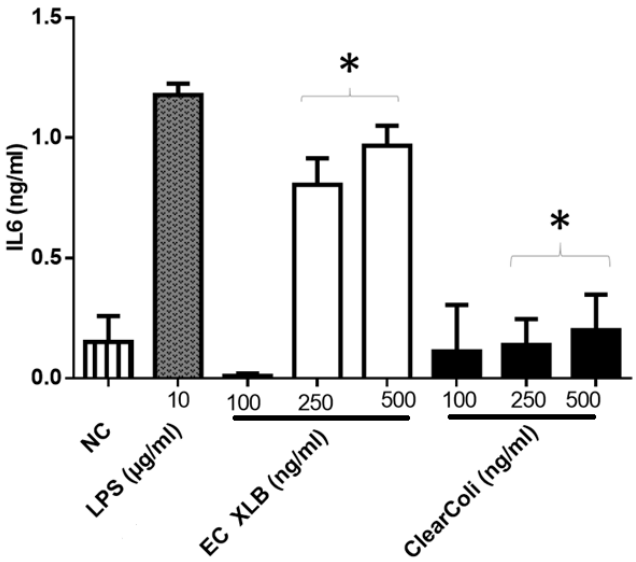


Figure 3.1 PfHsp70-1 expressed from *E. coli* ClearColi BL21 do not induce cytokine release on Macrophages cells
Wild-type murine Macrophages successfully stimulated with the indicated amount of LPS to induce cytokine production, untreated Macrophages (NC) were cultured in media alone. Cells cultured with PfHsp70-1 purified from *E. coli* ClearColi BL21 (ClearColi) failed to stimulate cytokine IL6 as compared to cells cultured with PfHsp70-1 produced from *E. coli* XL1 Blue (EC XLB) expression host. Student t-test was used to demonstrate statistical differences (*$P \leq 0.00143$).

Similarly, LPS was used to induce IL8 production on TLR4 transfected macrophage cells, while cells cultured in media alone exhibited significantly low IL8 expression (Figure 3.2). TLR4 transfected cells seeded with PfHsp70-1 purified from *B. choshinensis* exhibited significantly low ($P < 0.00$) IL8 expression (Figure 3.2). However, TLR4 transfected cells co-incubated with the same concentration of PfHsp70-1 purified *E. coli* XL1 Blue exhibited a high IL8 cytokine expression (Figure 3.2).

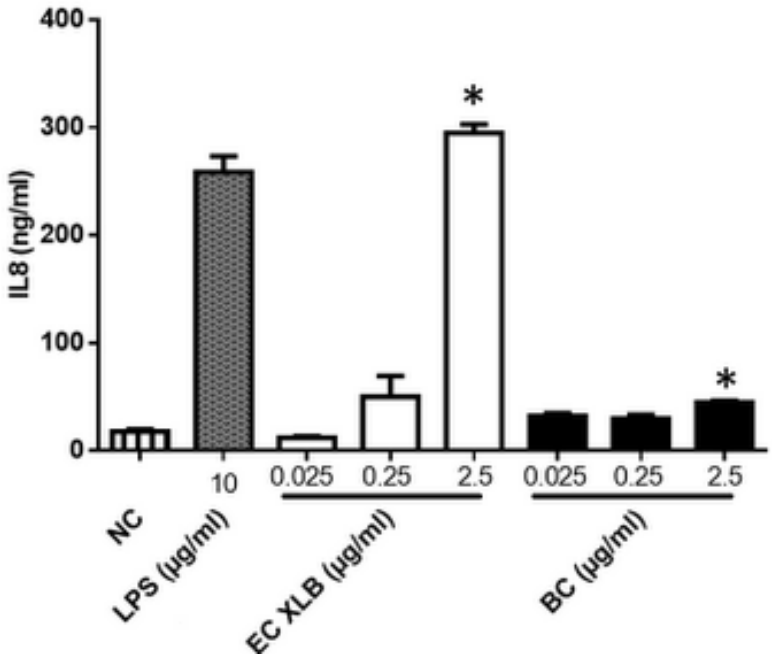


Figure 3.2 PfHsp70-1 expressed from *B. choshinensis* does not induce cytokine release on wild type Macrophages
TLR4 transfected HEK293 cells co-cultured with the indicated amount of LPS to induce cytokine production, untreated macrophage cells (NC) were cultured in media alone. Cells cultured with PfHsp70-1 produced using *B. choshinensis* failed to stimulate IL8 induction as compared to cells cultured with PfHsp70-1 purified from and *E. coli* XL1 Blue cells. Statistical differences were calculated using Student t-test (*$P < 0.0001$).

Both TLR2 and TLR4 have been suggested to be involved in Hsp70 mediated signalling (Asea *et al*., 2002). This study found that macrophages seeded with PfHsp70-1 purified from *E. coli* XL1 Blue expression host, activated the macrophages to express cytokine production and whereas, PfHsp70-1 produced in *B. choshinensis* could not induce cytokine release (Figure 3.2 and Figure 3.3). Even at fairly high concentration (10 µg/ml), PfHsp70-1

produced using *B. choshinensis* could not induce cytokine release (Figure 3.3). Taken together, these results indicate that the observed stimulation originated from the residual LPS in the protein preparation produced in *E. coli* XL1 Blue cells.

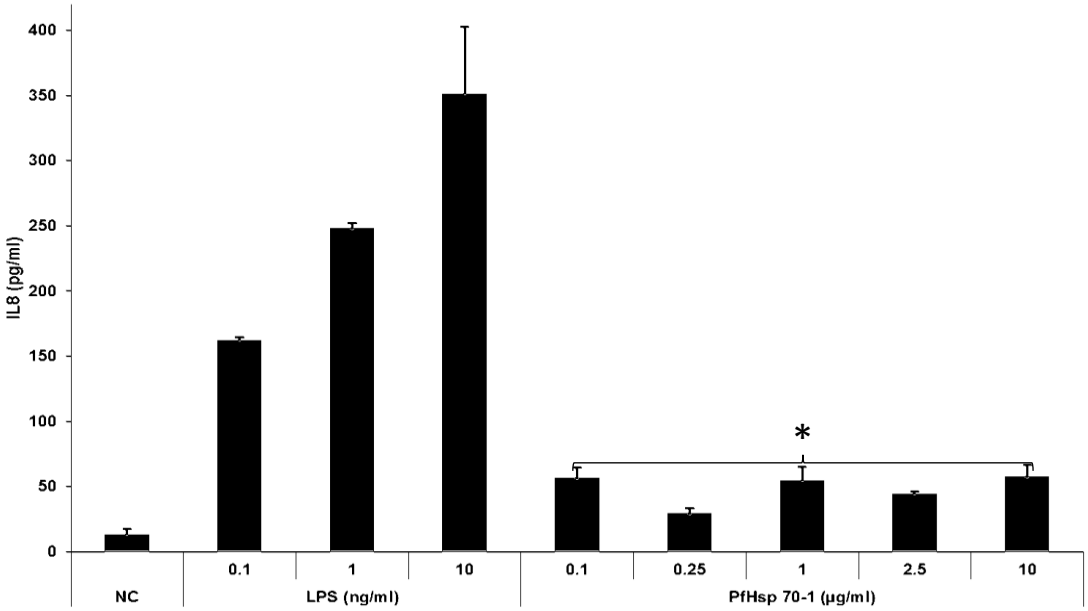


Figure 3.3 Assesment of the effect of PfHsp70-1 on cytokine release by murine macrophages
TLR4 transfected HEK293 cells were successfully stimulated with the indicated amount of LPS and untreated macrophages (NC) were used as a negative control. Cells cultured with the indicated amount of PfHsp70-1 purified from *B. choshinensis* failed to stimulate cytokine release, even at high concentrations of PfHsp70-1. One-way ANOVA was used to statistical analysis (*$P \geq 0.5$).

3.3.2 Effect of polymyxin-B on the cytokine stimulation activity of recombinant PfHsp70-1

Lipopolysaccharides (LPS) are gram negative endotoxins that form a major component of the Gram-negative bacterial cell wall. LPS are pyrogenic molecules that co-purify with recombinant protein from *E. coli* protein purification systems. Polymyxin-B is a peptide antibiotic that is widely used to neutralise the cytokine stimulation activity of LPS on co-purified with recombinant proteins (Cardoso *et al*., 2007). Hence, it was important to investigate the effect of polymyxin-B on cytokine release by co-culturing cells treated with PfHsp70-1 in the absence and presence of polymyxin-B. As expected macrophages incubated

with medium alone or medium treated with polymyxin-B remained unstimulated. Murine macrophages treated with LPS, successfully stimulated the production of IL6 and IL12. However, in the presence of polymyxin-B, LPS stimulation was significantly inhibited (Figure 3.4 A and B).

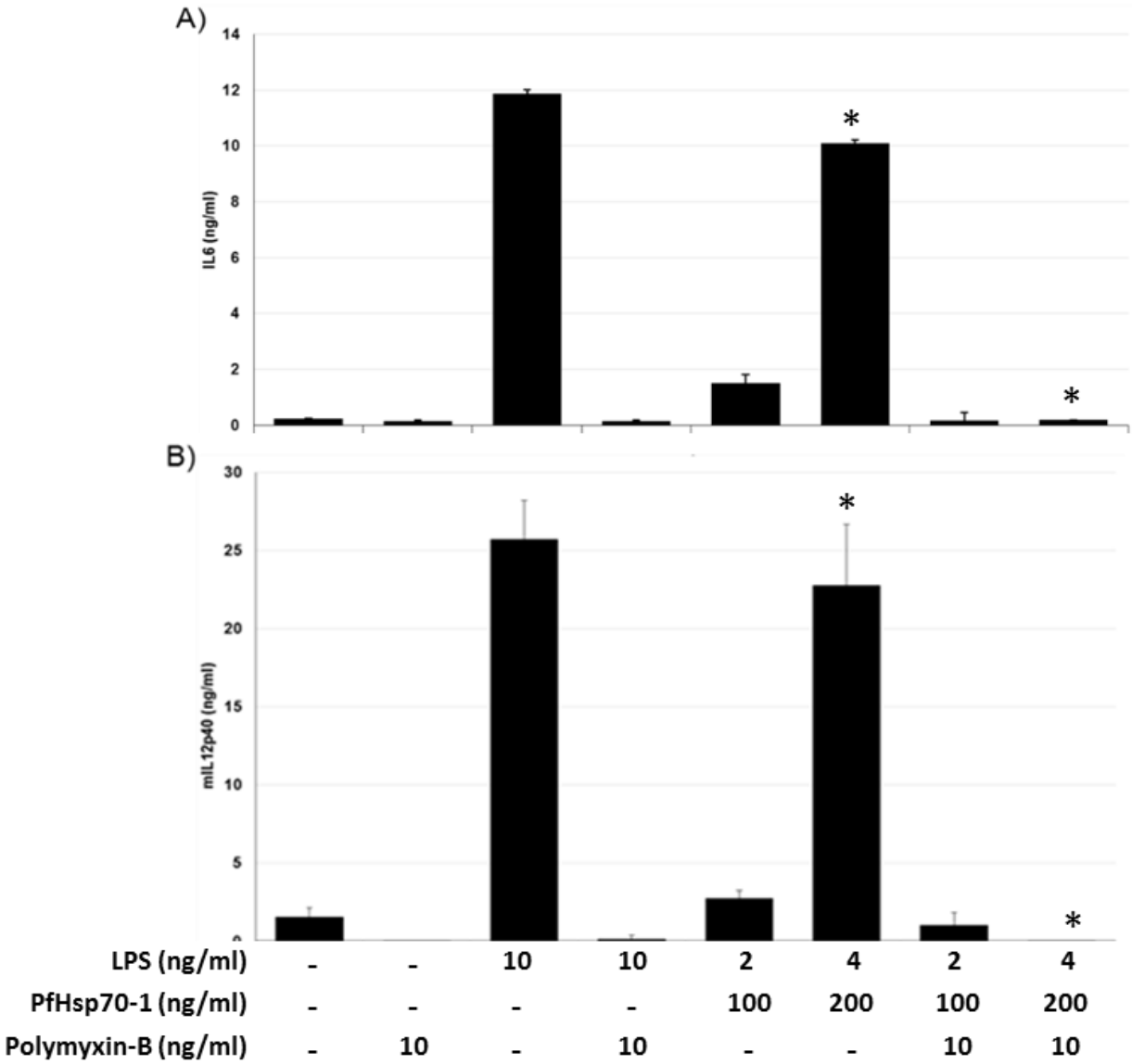


Figure 3.4 Polymyxin-B suppresses cytokine stimulation activity of LPS contaminating recombinant PfHsp70-1

PfHsp70-1 purified from *E. coli* XL1 Blue and LPS successfully stimulated IL6 (A) and mIL12p40 (B) production by murine dendrite cells. However, in the presence of 10 ng/ml of polymyxin-B cytokine induction was reduced. M, represents untreated cells grown in culture media alone. Student t-test was utilised to show statistical differences (*$P < 0.0001$).

Macrophages incubated with PfHsp70-1 prepared from *E. coli* XL1 Blue cells, successfully simulated IL6 and IL12 induction by the macrophage cells (Figure 3.4 A and B). On the other hand, cells incubated with PfHsp70-1 in the presence of polymyxin-B failed to produce IL6 and IL12 (Figure 3.4 A and B). These results indicate that the addition of polymyxin-B directly inhibits cytokine induction. Furthermore, these results strongly suggest that the observed stimulation in the absence of polymyxin-B are derived from the residual LPS contaminating the protein.

3.3.3 Protein structural integrity is required for PMN activation

Heat inactivation of proteins by boiling is often employed to validate the observed effects of protein preparation on immune stimulation (Kakimura *et al*., 2003). To examine the impact heating on LPS ability to activate PMNs; various concentrations (0.01, 0.04, 0.2 and 1 ng/ml) of LPS were boiled at 100°C for 10 minutes. After boiling the LPS was left to cool and seeded with PMNs at the indicated concentrations; activation was monitored by observing PMN reduce L-selectin shedding and IL8 production. As expected PMN seeded with boiled LPS preparations also appeared to reduce L-selectin shedding and IL8 production by PMNs, in a similar dose dependant manner as exhibited by non-boiled LPS preparations (Appendix B; Figure B.1). These results suggest that LPS modulatory stimulation on PMNs were not altered by boiling. The findings noted in this study are in line with previous reports that LPS is heat stable (Aosai *et al*., 2006). This study, therefore, examined the dependency of functional protein immune PMN cell activation. PMNs were co-cultured with equal concentrations of boiled and a non-boiled PfHsp70-1_{NBD}. Non-boiled PfHsp70-1_{NBD} preparations successfully stimulated L-selectin shedding and IL8 production by PMNs; on the other hand, boiled fractions of the same protein exhibited reduced PMNs stimulation

(Figure 3.4 A and B). Heat-denatured PfHsp70-1_{NBD} samples lost the ability to stimulate PMN activation compared with intact non-boiled protein (Figure 3.4).

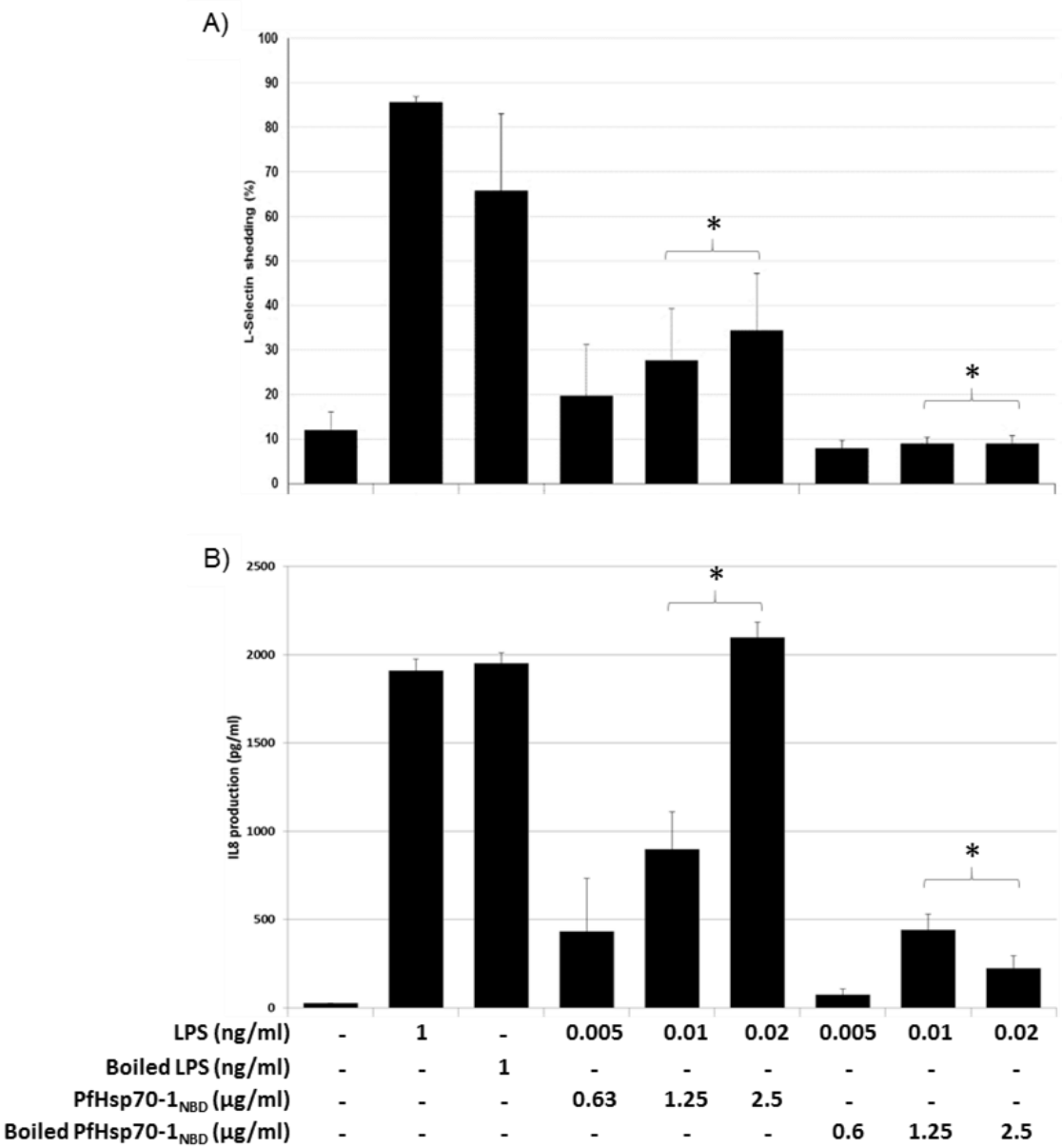


Figure 3.5. PfHsp70-1_{NBD} promotes L-selectin shedding and cytokine expression
Freshly isolated PMNs cultured with equal amounts of boiled and non-boiled LPS successfully induced cytokine expression. PfHsp70-1_{NBD} expressed from *E. coli* XL1 Blue contaminated with the indicated amounts of LPS. L-selectin shedding (A) and IL8 release (B) by PMNs were significantly reduced when cultured with boiled PfHsp70-1_{NBD} as compared to PMNs cultured with non-boiled PfHsp70-1_{NBD}. Student t-test was used to calculate statistical analysis (*$P \leq 0.05$).

These results appear to indicate that the observed PMN stimulation was not solely due to LPS co-purified with the protein. Moreover, that the cell stimulation may have been due to the aggregation prone PfHsp70-1_{NBD} protein.

3.4. Discussion

Innate immunity cells, such as macrophages and neutrophils are readily stimulated by the presence of endotoxins, even in minute quantities (Mader *et al.,* 2004). To test the immunogenicity of recombinant proteins produced using *B. choshinensis* and *E. coli* ClearColi BL21expression host system, PfHsp70-1 produced from these cells were co-cultured for 18 hours with macrophages. Findings from this study revealed that PfHsp70-1 purified from *B. choshinensis* and *E. coli* ClearColi BL21cells failed to activate macrophages cells, on the other hand, PfHsp70-1 produced using *E. coli* XL1 Blue cells successfully stimulated the immune cells. Results from this study suggest that *E. coli* ClearColi BL21and *B. choshinensis* are reliable protein expression machinery that can produce recombinant protein without immunogenic LPS (Mamat *et al*., 2013). The *E. coli* ClearColi BL21 strain used in this study has a modified LPS (Lipid IV), as compared to LPS in wild-type *E. coli* strains (section 2.1; Mamat *et al*., 2013). Hence, recombinant PfHsp70-1 produced from *E. coli* ClearColi BL21does not activate an endotoxic immune response. This study present data for the first time that malaria heat shock proteins can be purified without LPS by using the appropriate bacterial host.

Contrary to previously published data by Qazi and colleagues (2005) PfHsp70-1 expressed without residual LPS did not induce cytokine expression. However, it should be noted that PfHsp70-1 used by Qazi and colleagues (2005) was purified from *E. coli* strain BL21 (DE3) protein expression host and may have had traces of LPS co-purified with the protein. In this study PfHsp70-1 purified from the *E. coli* XL 1 Blue cells was passed through the endotoxin removal column, to remove LPS content (section 2.3.10). However, the residual trace amounts of LPS on the PfHsp70-1 prepared from *E. coli* XL1 Blue cells, were potent enough to activate the immune cells and induce cytokine expression. Intriguingly, when the same

protein was co-cultured with Macrophages cells in the presence polymyxin-B, PfHsp70-1 failed to stimulate the immune cells. Polymyxin-B has traditionally been used to nullify the effects of LPS in immunology experiments (Tynan *et al*., 2012). This suggests that the residual LPS contaminating the LPS preparation may have induced the immunomodulatory response. Alternatively PfHsp70-1 may require LPS to stimulate the immune cells.

Luong and colleagues (2012) proposed that macrophages stimulation by recombinant Hsp70 requires active Hsp70 protein itself. PMNs cultured with equal levels of boiled and non-boiled PfHsp70-1_{NBD} protein prepared from *E. coli* XL1 Blue. Boiled PfHsp70-1_{NBD} did not stimulate cytokine production whereas non-boiled PfHsp70-1_{NBD} successfully stimulated cytokine response. Moreover, LPS-free PfHsp70-1_{NBD} also did not exhibited cytokine stimulation activity (Appendix B; Figure B.5). Misra and Ramachandran (2009) showed that PfHsp70-1_{NBD} is highly susceptible to thermal degradation and aggregation. Furthermore, the same authors demonstrated that PfHsp70-1_{NBD} requires its PBD (section 1.4.2) to exhibit thermal stability and chaperone activity. Joubert and colleagues (2012) showed that aggregated or partially misfolded proteins also stimulate cytokine induction from immune cells. Furthermore, the immunogenicity of proteins depends on the structure and orientation of the protein (Hermeling *et al*., 2005; Joubert *et al*., 2012). In addition, aggregated proteins have been shown to stimulate cytokine expression by interacting with TLR4 and TLR2 on immune cells (Joubert *et al*., 2012). It is thus possible that the observed stimulation may have been due to the aggregation prone PfHsp70-1_{NBD} protein and not necessarily due to the chaperokine role of the protein (Hermeling *et al*., 2005; Joubert *et al*., 2012). Boiling PfHsp70-1_{NBD} may have altered the orientation of the aggregated protein, hence, the reported loss of immunogenicity. In light of this, since PfHsp70-1 has been confirmed in the blood of malaria infected patients (Joshi *et al*., 1992; Kappes *et al*., 1993). It is, therefore, conceivable

that if fragments of PfHsp70-1 were to trickle into the blood-stream, they would similarly be immunogenic.

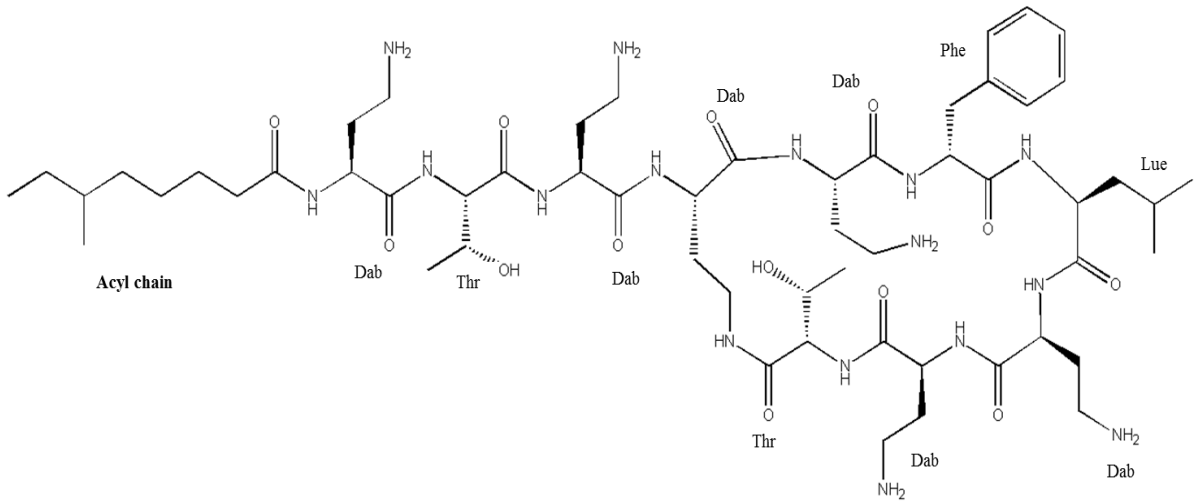
Taken together this data appears to suggest that cytokine stimulation in this report may largely be due to the presence of LPS contamination or protein aggregation. Results from this study have demonstrated that functional PfHsp70-1 does not possess immunomodulatory activity, *in vitro*. Polymyxin-B was successfully used to nullify the effect of the residual LPS on recombinant protein, however, the effects polymyxin-B on the structural and functional intergrity of PfHsp70-1 have not been documented. Hence, Chapter 4 investigates the interaction between PfHsp70-1 and polymyxin-B, *in vitro*.

CHAPTER 4

EVALUATING THE EFFECT OF POLYMYXIN-B ON THE STRUCTURAL AND FUNCTIONAL INTERGRITY OF PFHSP70-1

4.1 Introduction

Polymyxin-B is an established antibiotic that has recently been highlighted as a potential drug treatment for multidrug-resistant pathogens (Zavascki *et al.*, 2007). Initially, the administration of polymyxin-B was withdrawn due to its adverse effects on the renal and neurological systems (Kallel *et al*., 2005). However, the emergence of bacterial multidrug-resistance strains towards currently available antibiotics has led to the reuse of 'old' antibiotics such as polymyxin-B (Berlana *et al*., 2005; Zavascki *et al.*, 2007). Polymyxin-B is a naturally occurring cationic cyclic lipopeptide, isolated from *Bacillus polymyxa* (Kallel *et al*., 2005). Polymyxin-B consists of a positively charged cycloheptapeptide ring and a hydrophobic tail (Figure 4.1). The hydrophobic tail comprises of an acyl chain with eight or nine carbons attached through an amide bond (Figure 4.1; Domingues *et al*., 2012). The cationic peptide in polymyxin-B is essential for binding to lipid-A of LPS. LPS (section 2.1) are cyclic positively charged peptide antibiotics linked to a fatty acid residue (Landman *et al*., 2008).



Polymyxin-B is a positively charged molecule. The positive charges on the structure are conferred by diaminobutyric acid (Dab) residues on the cycloheptapeptide and the N-terminal region. Phenylalanine (Phe) and leucine (Leu) confer the hydrophobic locus to the polymyxin-B structure; The N-terminal region also contains a hydrophobic tail made of an acyl chain with C-8 or C-9 fatty acids. Co-ordinate files of the polymyxin-B structure were obtained from Nature Chemical Biology (www.nature.com/nchembio/journal/v5/n7/compound/nchembio.184_comp4). The structure was then visualized using ChemDraw Ultra®, Version 7.0.1 (CambrigeSoft).

Polymyxin-B is unique among most antibiotics as it has both bactericidal and anti-endotoxic properties. Polymyxin-B has a bactericidal effect on Gram-negative bacteria, the cationic peptide in polymyxin-B binds to lipid-A on the LPS. This interaction allows the polymyxin-B hydrophobic tail to penetrate through the bacterial outer membrane, causing the bacterial membrane to destabilize and rupture (Tsubery *et al*., 2000). Domingues and coworkers (2012) suggested that polymyxin-B interacts with LPS electrostatically and promotes LPS aggregation. Through dynamic light scattering experiments, polymyxin-B was seen to induce LPS aggregation in a concentration-dependent manner (Domingues *et al*., 2012). The LPS aggregation induced by polymyxin-B is believed to be part of LPS neutralisation mechanism thereby inhibiting the interaction of LPS with cell receptors, such as TLR4 (Domingues *et al*., 2012). Hence, LPS contaminated products lose their endotoxicity after treatment with polymyxin (Domingues *et al*., 2012).

Minawaga and colleagues (2012) presented evidence that cyclic lipopeptide antibiotics, such as polymyxin-B and colistin may bind to NBD of prokaryotic Hsp90 (HtpG) to interfere with the chaperone activity of the protein. Using surface plasmon resonance (SPR) and protein aggregation suppression activity assays polymyxin-B was shown to interact physically with HtpG and directly interfered with its chaperone role (Minawaga *et al*., 2012). Polymyxin-B has routinely been used to remove and neutralize the effects of LPS from recombinant protein studies (Saraswat *et al*., 2013). Most recombinant proteins are prepared from an *E. coli* expression host, thereby, harbouring LPS contaminants in the final protein products. Therefore, it is likely that the use of polymyxin-B, as an endotoxin neutralizer for recombinant products or proteins, may have an undesirable consequence. As polymyxin-B

may potentially be bound by chaperone proteins thus interfering with their chaperokine roles (section 3.3.2).

Like other Hsp70 chaperones, PfHsp70-1 is thought to bind to nascent and misfolded peptides (Shonhai *et al*., 2007; Shonhai *et al*., 2008). PfHsp70-1's aggregation suppression activity has previously been described using a well-optimized *in vitro* assay. The assay is based on the Hsp70's chaperone capability to prevent aggregation of a protein that is prone to aggregation, such as MDH (Goloubinoff *et al*., 1999; Basha *et al*., 2004). Hsp70 proteins typically prevent protein aggregation by binding to hydrophobic segments of misfolded proteins (Mayer and Bukau, 2005; Kabani and Martineau, 2008). Since, polymyxin-B is a hydrophobic positively charged peptide it is likely that PfHsp70-1 may bind to polymyxin-B. This interaction may also affect PfHsp70-1's chaperone function, leading to the inhibition of *P. falciparum* parasite growth (Shonhai *et al*., 2007; Pesce *et al*., 2010). This study, therefore, sought to investigate the effect of polymyxin-B on the chaperone role of PfHsp70-1.

The specific objectives of the study included:

1) Investigating the effect of polymyxin-B treatment on PfHsp70-1 thermal stability

2) Exploring the effect of polymyxin-B on PfHsp70-1 structural conformation using limited proteolysis and native-PAGE analysis

4.2 Experimental procedure

4.2.1 Materials

Polymyxin-B sulphate (Duchefa, Amsterdam), proteinase-K and MDH (both from Sigma, U.S.A). The rest of the reagents used in the study are listed in (Table D.1; Appendix D).

4.2.2 Investigating the effect of polymyxin-B on the thermal stability of PfHsp70-1

PfHsp70-1's chaperone role has been demonstrated through its ability to suppress the heat-induced aggregation of MDH (Shonhai *et al*., 2008). PfHsp70-1 has been shown to be a fairly heat-stable molecule (Misra and Ramachandran, 2009). MDH is a highly unstable polypeptide that easily aggregates when subjected to harsh temperatures, ≥42°C (Shonhai *et al*., 2008; Tomoyasu *et al*., 2010). It has further been reported that the peptide binding domain (PBD) of PfHsp70-1 is necessary for the stability of the protein (Misra and Ramachandran, 2009). The current study investigated the effect of polymyxin-B on the stability of PfHsp70-1, when subjected to thermal stress at 42°C. The assay was conducted as follows; assay buffer (20 mM Tris, pH 7.4; 100 mM NaCl) was prepared to a final concentration of PfHsp70-1 (1.3 mM) and/or MDH (0.65 M) in the presence of polymyxin-B, following a method described previously (Shonhai *et al*., 2008; Luthuli *et al*., 2012). The aggregation of MDH alone or PfHsp70-1 alone and PfHsp70-1 with MDH at 42°C was investigated by measuring at an absorbance of 360 nm for 30 minutes.

To further confirm the ability of PfHsp70-1 to suppress heat-induced aggregation of MDH in the absence and presence of polymyxin-B, a previously described method was followed (Luthuli *et al*., 2012; Makumire *et al*., 2014). Protein samples were reconstituted in 20 µL final volume in assay buffer, containing 1.3 µM PfHsp70-1 alone and 1.3 µM PfHsp70-1 with

1μM MDH final concentrations were preheated to 42°C for 5 min in the presence of 2 mM polymyxin-B. Control experiments were set up in the absence of polymyxin-B. The reaction was incubated at 42°C for 30 min. The protein solubility was investigated by centrifugation for 10 min at 14 000 rpm. The remaining pellet was resuspended in SDS-PAGE loading buffer. The protein samples were boiled for five min before analysis by SDS-PAGE and subsequently detected by Western blot analysis using α-PfHsp70-1 antibody (Pesce *et al*., 2008).

4.2.3 Investigating the effect of polymyxin-B on the structure of PfHsp70-1 using limited proteolysis

Limited proteolysis experiments can be used to investigate conformational features of proteins (Fontana *et al*., 2004; Banijamali *et al*., 2013). Proteinase-K has been shown to exhibit a very broad cleavage specificity (Cabrera *et al*., 2002), hence can easily digest any exposed proteolytic loops due to ligand-induced conformational changes. Limited proteolysis was conducted to investigate the effect of polymyxin-B on the structure formation of PfHsp70-1. Purified PfHsp70-1 (2 µg/ml) treated with and without polymyxin-B (0.5 ng/ml) were digested by proteinase-K [prepared in sterile phosphate buffered saline (pH 7.4)] at 37°C. Samples were taken at various time intervals (0, 5, 15, 30, 45 min) and analysed by SDS-PAGE.

4.2.4 Investigating the effect of polymyxin-B on PfHsp70-1 using native-PAGE

Polyacrylamide gel electrophoresis under native conditions (native-PAGE) is a well-established and method for probing ligand-induced structural changes. Native-PAGE is a “non-denaturing" gel electrophoresis that allows the proteins to be resolved in their native conformation (Chan *et al*., 2012). Thus, native-PAGE can be used to study protein folding

conformation, self-association and aggregation (Hubbard, 1998; Chan *et al*., 2012). To investigate ligand specific interaction between polymyxin-B and PfHsp70-1, native PfHsp70-1 (40 µg/ml) was treated with ADP (10 mM), ATP 10 mM) and polymyxin-B (6.25 µg/ml) for 5 min at room temperature. Untreated native PfHsp70-1 (40 µg/ml) was used as a negative control. Samples were then resolved on 4% native-PAGE.

4.3 Results

4.3.1 Polymyxin-B promotes PfHsp70-1 aggregation

PfHsp70-1 has previously been shown to be stable and functional at 48°C (Shonhai et al., 2008). Misra and Ramachandran (2009) demonstrated that PfHsp70-1_{NBD} is thermally stable up to 50°C. This study, therefore, sought to investigate the effect of polymyxin-B on the thermo-stability of PfHsp70-1. To test whether PfHsp70-1 remained heat stable when challenged with polymyxin-B, the proteins were suspended in assay buffer and then exposed to heat at 42°C for 30 minutes. No significant turbidity increase was observed when the untreated protein was exposed to heat confirming that the purified PfHsp70-1 used in this study was heat stable. However, increase in turbidity readings were observed when the PfHsp70-1 was exposed to in the presence of various concentrations of polymyxin-B (6.25, 25, 50 µg/ml) at 42°C (Figure 4.2). Turbidity increase was observed in a dose-dependent manner (Figure 4.2). The increase in turbidity was attributed to PfHsp70-1 aggregation, since the control experiment in which polymyxin-B was heated 42°C did not register notable increases in turbidity.

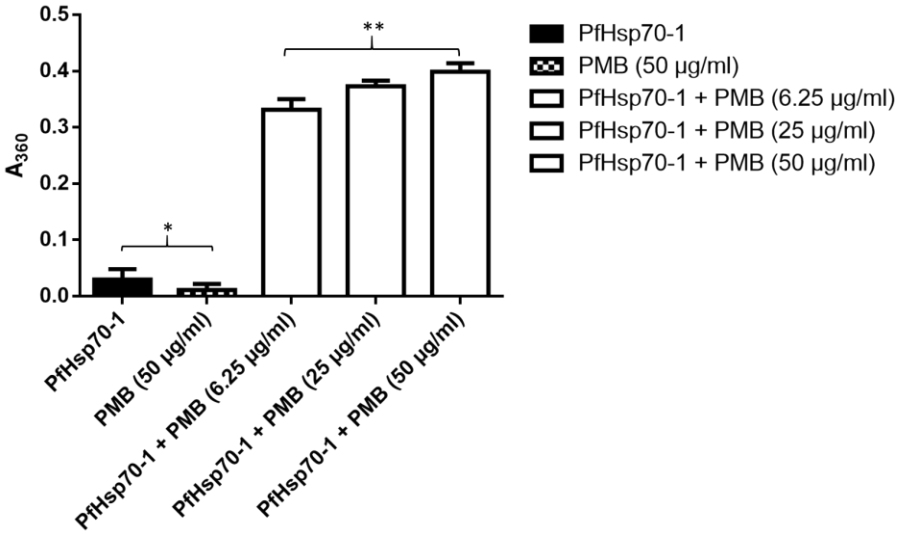


Figure 4.2 Polymyxin-B promotes PfHsp70-1 destabilisation
PfHsp70-1 thermoro-stability was reduced in a dose-defendant manner. Untreated PfHsp70-1 was used as control. PfHsp70-1 concentration was kept constant at 1.5 µM, the aggregation of denatured PfHsp70-1 was measured at 360 nm over 30 min. Various concentrations (6.25, 25, 50 µg/ml) of polymyxin-B incubated with PfHsp70-1 at 42°C, absorbance was monitored at 360 nm. Student's t-

test was used to calculate the statistical significance. *P = 5.12, **$P \leq 0.00972$. PMB, represents polymyxin-B.

4.3.2 Polymyxin-B interferes with the chaperone function of PfHsp70-1 *in vitro*

MDH is an aggregation-prone protein which is ideal to study the influence polymyxin-B has on the chaperone function of PfHsp70-1, *in vitro*. In the current study, the effect of polymyxin-B on PfHsp70-1 aggregation suppression activity was investigated at 42°C. Purified PfHsp70-1 protein was verified to be heat stable before evaluating its ability to suppress MDH aggregation. Control experiment showed that when PfHsp70-1 was incubated at 42°C, it protein did not aggregate due to heat stress (Figure 4.3 B) and subsequently remained in soluble form (Figure 4.3 A, lane S1). Polymyxin-B challenged by heat also did not show notable aggregation (Figure 4.3 B). On the other hand, exposing MDH (0.65 µM) to 42°C, resulted in increased turbidity (0.33 absorbance units) over the 30 minutes incubation (Figure 4.3 B). Turbidity increase was observed when MDH was heated alone; thus, the observed results were attributed to MDH aggregation.

To evaluate whether the PfHsp70-1 used in this study was able to prevent heat-induced aggregation, PfHsp70-1 and MDH were suspended in assay buffer and then exposed to heat at 42°C for 30 minutes. As expected, PfHsp70-1 incubated with MDH remained in soluble form when subjected to heat at 42°C; only a small fraction was seen in the pellet fraction by Western analysis (Figure 4.3, lanes P3, S3). No significant turbidity increase was observed in the reaction tube inn which PfHsp70-1 was incubated with MDH (Figure 4.3 A). Suggesting that PfHsp70-1 suppressed the thermal aggregation of MDH. Interestingly, in the presence of polymyxin-B, PfHsp70-1 and MDH proteins were observed in the pellet fraction (Figure 4.3, lanes P4, S4). An increased turbidity (approximately 0.32 absorbance units) was observed in the reaction tube were PfHsp70-1 was incubated with MDH in the presence of polymyxin-B

(Figure 4.3 B). Taken together this results suggest that polymyxin-B inhibits the chaperone activity of PfHsp70-1.

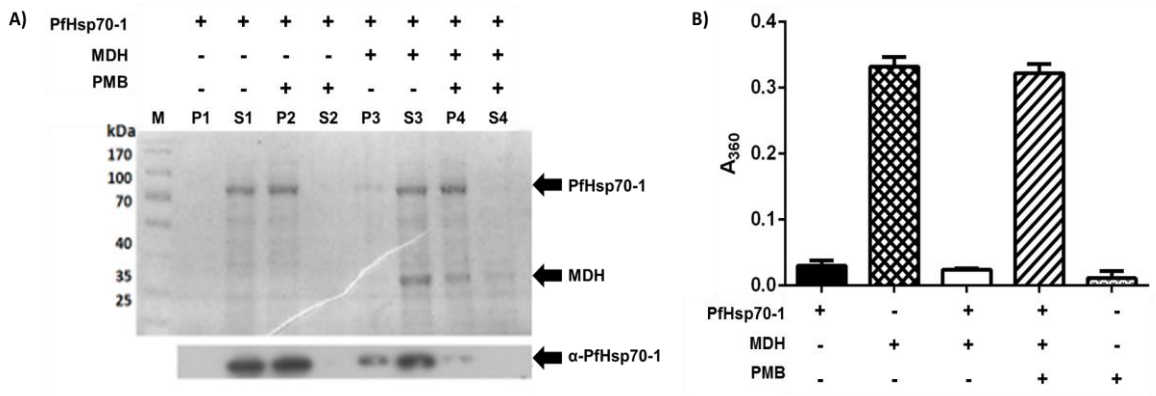


Figure 4.3 Polymyxin-B induces the aggregation of PfHsp70-1
PfHsp70-1 successfully suppressed the aggregation of MDH. However, in the presence of polymyxin-B PfHsp70-1 failed to prevent the aggregation of MDH. (A) SDS-PAGE (upper panel) and α-PfHsp70-1 polyclonal antibody (1:2000) was used to confirm the presence of the proteins (lower panel). The letters 'P' and 'S' represent pellet and soluble fraction, respectively. M, protein molecular weight marker of recombinant proteins. (B) MDH was added to the assay buffer to initiate the reaction and produce a bar graph representative of aggregation of MDH alone. The '+' symbol represent components present in the reaction whereas, the '-' represent those that were absent. PMB, represents polymyxin-B.

4.3.3 PfHsp70-1's susceptibility to proteolysis is enhanced in the presence of polymyxin-B

Limited proteolysis is widely used to explain globular protein folding and probe their conformational features (Cabrera *et al*., 2002; Banijamali *et al*., 2013). Since proteinase-K cleaves and digest the exposed protein loops, this study sought to investigate the effect polymyxin-B has on PfHsp70-1's structural conformation and folding. Purified PfHsp70-1 treated with and without polymyxin-B were digested with 0.5 ng/ml of proteinase-K, over a time-course (0, 5, 10, 15, 30, and 45 min) at 37°C. As expected PfHsp70-1 protein incubated in the presence of proteinase-K over the time-course generated digestion fragments of 35 kDa. However, it was noted that in the presence of polymyxin-B, PfHsp70-1 generated digestion fragments of 35 kDa at a higher rate as compared to PfHsp70-1 digested with proteinase-K in the absence of polymyxin-B treatment (Figure 4.4). The disappearance of the

full length PfHsp70-1 band around the 70 kDa was observed at a faster rate on protein digested in the presence of polymyxin-B as compared to PfHsp70-1 digested in the absence of polymyxin-B treatment (Figure 4.4). This data suggest that polymyxin-B interacts with PfHsp70-1 and modifies its structural conformation, thereby making PfHsp70-1 easily susceptible to proteolysis degradation.

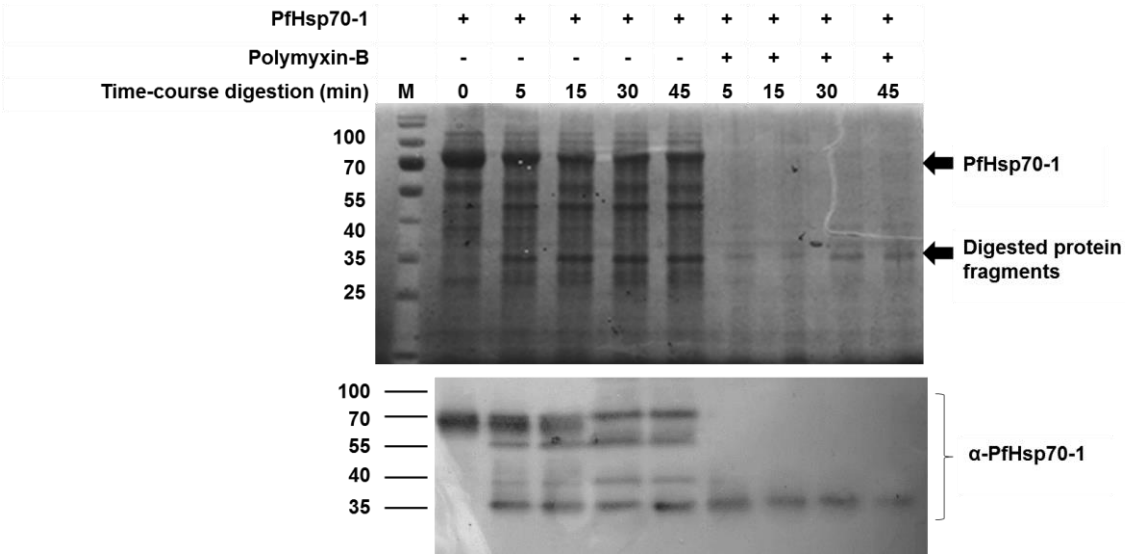


Figure 4.4 Partial proteolysis analysis of recombinant PfHsp70-1 digested in the presence and absence of polymyxin-B
Comparison of the proteolytic profiles of proteinase-K digested PfHsp70-1 incubated with and without the presence of polymyxin-B. The '+' symbols represent components present in the reaction whilst '-' represent those that were absent. Proteolytic products were resolved by 12.5% SDS-PAGE (upper panel) and confirmed by Western blot (lower panel) using α-PfHsp70-1 to confirm the presence of PfHsp70-1. Lane M, molecular weight marker. Lane 0, untreated protein PfHsp70-1, lane 5, 15, 30 and 45 represent 5, 15, 30 and 45 min time-course proteinase-K digestion.

4.3.4 Polymyxin-B's interaction with PfHsp70-1 induces a mobility shift on Native-PAGE analysis

Native-PAGE is a powerful tool that is commonly used to visualise interactions between proteins and interacting molecules (Ryder *et al*., 2008). This assay utilizes a simple gel mobility shift to resolve ligand-induced structural changes. Native PfHsp70-1 was used to study the interaction between polymyxin-B and PfHsp70-1. ADP has previously been shown not to induce minimal on the structural conformation of Hsp70 protein (Kim et al., 2012).

Therefore, untreated PfHsp70-1 and ADP treated protein, were used as negative controls and as expected, exhibited a lower and higher band migration when resolved by native-PAGE (Figure 4.5). This results represents that in the absence of any conformational change native PfHsp70-1 migrates with these two distinct bands (see arrow; Figure 4.5). On the other hand, PfHsp70-1 treated with ATP, exhibited a mobility shift with distinctive lower singular band migration. ATP is known to induce a conformational change on PfHsp70-1. The change in PfHsp70-1 band migration was attributed to structural changes induced by the structural interaction between PfHsp70-1 and ATP. Interestingly, PfHsp70-1 challenged with polymyxin-B similarly exhibited mobility band shift when analysed by native-PAGE (Figure 4.5). Taken together this data suggests that PfHsp70-1 interacts with polymyxin-B, thereby inducing a conformational change. Hence, the observed band mobility shift. Western analysis was used to confirm PfHsp70-1 migration (lower panel; Figure 4.5).

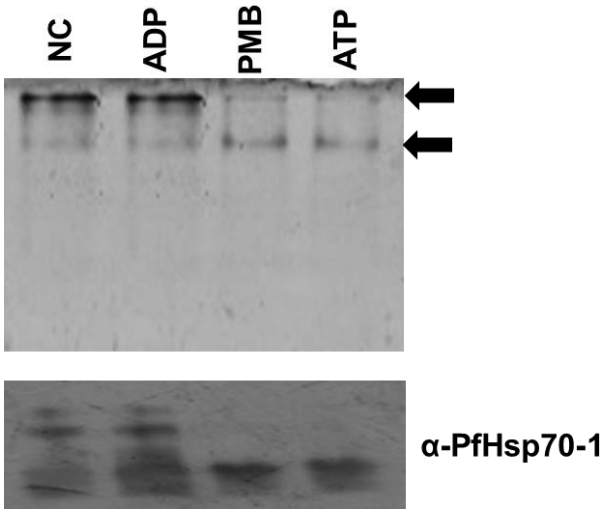


Figure 4.5 Influence of polymyxin-B on PfHsp70-1 native-PAGE migration
Polymyxin-B and ATP addition, respectively promote increased migration of PfHsp70-1. Lane ADP, PMB and ATP represent PfHsp70-1 treated with ADP, polymyxin-B and ATP, respectively. Lane NC, untreated PfHsp70-1 used as a negative control. The arrow on the right hand side represents mobility shifts of native PfHsp70-1. Native-PAGE was visualised with Coomassie blue, and α-PfHsp70-1 antibody was used to confirm the presence of PfHsp70-1.

4.4 Discussion

PfHsp70-1 is a ubiquitous, highly conserved protein that is expressed throughout all the intra-erythrocytic stages of the malaria infection (Aurrecoechea *et al*., 2009). Although PfHsp70-1's is constitutively expressed, it has been shown to increase~~s~~ after heat shock, thus is crucial for the survival of the parasite (Kumar *et al*., 1991). Hence interfering with PfHsp70-1's chaperone role is important in reducing the pathogenicity of the disease. The study, therefore, evaluated the possible interaction between polymyxin-B and PfHsp70-1, a potential selective drug target (Pesce *et al*., 2010; Shonhai, 2010; Shonhai *et al*., 2014).

Minawaga and co-workers (2012) demonstrated that polymyxin-B interacts with HtpG and interferes with its chaperone function. Findings from this study revealed that in the presence of polymyxin-B, PfHsp70-1 exhibits reduced ability to suppress MDH induce thermal aggregation. Moreover, the thermo-stability of PfHsp70-1 was disrupted by increasing concentrations of polymyxin-B. Means of limited proteolysis were employed to investigate PfHsp70-1's interaction with polymyxin-B. In this study, PfHsp70-1 treated with polymyxin-B was readily digested by proteinase-K as compared to PfHsp70-1 with protein. Implying that the interaction with between PfHsp70-1 polymyxin-B induced a conformational change that exposed the PfHsp70-1 to proteinase-K digestion at an increased rate, as compared to PfHsp70-1 digested in the absence of polymyxin-B.

The interaction of between polymyxin-B and PfHsp70-1 was further confirmed using native-PAGE analysis. ATP binding to PfHsp70-1 has been shown to induce structural conformational changes on PfHsp70-1 (Shonhai *et al*., 2008). The present study revealed conformational changes as mobility shifts where observed when the PfHsp70-1 was analysed by native-PAGE. The polymyxin-B and ATP-induced conformational changes caused a

mobility shift to a singular distinctive band, whereas, untreated PfHsp70-1 or ADP-treated exhibited the protein migration shift, with a monomer and dimer species when subjected to native-PAGE. Taken together, these results reveal that polymyxin-B interacts with PfHsp70-1 resulting in the reduced chaperone function of PfHsp70-1. The current study cannot identify the domain through which polymyxin-B interacts with PfHsp70-1 nor the interaction mechanism between polymyxin and PfHsp70-1. However, it is likely that PfHsp70-1 may bind with polymyxin-B through its chaperone role since polymyxin-B is a hydrophobic peptide molecule. Further studies however are still required for to confirm if the interaction occurs through the PBD via the EEVD motif. Ramya and co-workers (2006) showed that the antimalarial compound, DSG, interacts with PfHsp70-1 though it's C-terminal EEVD motif subsequently inducing PfHsp70-1 precipitation, hence interferes with the chaperone role of PfHsp70-1 (Ramya *et al*., 2007). Polymyxin-B may bind to PfHsp70-1 through the C-terminal in the same manner DSG binds to PfHsp70-1.

Furthermore, Colistin sulfate salt was reported to increase the surface-exposed hydrophobicity of HtpG thus induce their protein oligomerisation (Minawaga *et al*., 2012). Although this study could not conclusively determine the domain that PfHsp70-1, this study however hypothesizes that the interaction of polymyxin-B with PfHsp70-1 may lead to exposure of the hydrophobic pocket of PfHsp70-1 (section 1.2.2.2). These structural changes may disrupt the chaperone site thereby interfering with the thermal stability of PfHsp70-1 and its chaperone activity. Compounds that physically interact with the chaperone activity of PfHsp70-1 have been shown to inhibit the growth of *P. falciparum* parasite culture (Chiang *et al*., 2009; Botha *et al*., 2011; Cockburn *et al*., 2011). Sharma and Biswas (2005) argued that the addition of polymyxin-B to *P. falciparum* cultures resulted in the reduction of protein kinase-C (PKC)-like activity comparable to addition of chloroquine to parasite cultures

(Sharma and Biswas, 2005). Recent findings from my collaborative partners revealed that polymyxin-B successfully inhibits the growth of asexual *P. falciparum* 3D7-infected erythrocytes, in a dose-dependent manner (unpublished data, manuscript in preparation). The mechanism by which polymyxin-B inhibits growth of asexual *P. falciparum* parasites is still unclear. DSG is a positively charged molecule, similar to polymyxin-B, DSG has been shown to interfere with the chaperone activity of PfHsp70-1. Due to its positive charge, DSG has been proposed to inhibit parasite growth by competing and disrupting positively charged nuclear-encoded apicoplast-targeted proteins being transported to the negatively charged pores on the apicoplast membrane (Ramya *et al*., 2007). It is therefore, conceivable that polymyxin-B may also inhibit the growth of *P. falciparum* in a similar manner as polymyxin-B is a highly positively charged peptide (Tsubery *et al*., 2000). However, further studies are needed to validate this mechanism of action.

In conclusion, these results demonstrate that polymyxin-B has antimalarial properties and can easily be adopted as a potential drug, since it is already an established drug which is available. This is the first report that proposes a direct antimalarial role of polymyxin-B on *P. falciparum* growth, *in vitro*. It should be noted that polymyxin-B drug resistance among gram-negative organisms has majorly been attributed to LPS modification. Synergism of polymyxins combined with other antimicrobials has been investigated (Ouderkirk *et al*., 2003; Wareham and Bean, 2006). Polymyxin-B drug combinations were found to be effective drug-combination therapeutic treatments (Ouderkirk *et al*., 2003; Wareham and Bean, 2006). Therefore, current antimalarial drugs can potentially be used in conjunction with polymyxin-B, as possible drug combinations to counter the looming antimalarial drug resistance.

CHAPTER 5

CONCLUSION AND FUTURE PROSPECTS

5.1. Conclusion and future work

The current study found that recombinant protein expression levels with *E. coli* Clearcoli and *Brevibacillus choshinensis* were relatively similar to PfHsp70-1 expressed in *E. coli* XL1 Blue. This study confirms that *E. coli* ClearColi BL21and *B. choshinensis s* expression are reliable and less laborious protein expression systems for producing proteins without LPS contamination. These LPS-free expression systems can be used for the rapid expression of LPS-free recombinant protein, which can be used in immunomodulatory studies. This study, therefore, recommends the expression of recombinant proteins in *E. coli* ClearColi BL21 and *B. choshinensis* cells for the production in the absence of LPS contamination. The results from this study suggest that PfHsp70-1 does not possess pro-inflammatory function, *in vitro*. Further studies are still required to evaluate PfHsp70-1's immunosuppressive role. Nonetherless, *in-vivo* studies are still required to fully elucidate the immnunomodulatory role of PfHsp70-1.

In addition, this current study presented evidence for the first time that polymyxin-B interacts with PfHsp70-1 and interferes with its chaperone function *in-vitro*. The study, also discusses polymyxin-B's ability to inhibit *P. falciparum* growth; setting it as a potential antimalarial therapeutic drug. Findings from this study, thus, warrant further investigation into PfHsp70-1 as a potential antimalarial drug target. It cannot be concluded from the present work that the observed antimalarial activity using parasite culture if it was due to direct PfHsp70-1 targeting by polymyxin-B. Engelbrecht and coworkers (2012) discussed various cell death mechanisms documented in *P. falciparum*. However, the findings in literature appear contradictory and undoubtedly little is known about exact cell death mechanisms and pathways in *P. falciparum*. Some studies, where *P. falciparum* cell death is induced by drugs or heat have been reported to

occur via programmed cell death while others report necrotic cell death (reviewed in Engelbrecht *et al*., 2012). Hence, it is difficult to speculate about the mechanisms by which polymyxin-B potentially causes death in malaria parasite-infected cells. However, a number of scenarios are plausible. As discussed in Chapter 1 (section 1.4.2), PfHsp70-1 is considered the major cytosolic Hsp70 in *P. falciparum* and has been found to be upregulated upon heat treatment of parasites. Thus, PfHsp70-1 is assumed to play a significant role in preventing heat-induced cell death of *P. falciparum* parasites. Hence, polymyxin-B's interaction with PfHsp70-1 may induce *P. falciparum* cell death. However, *in-vivo* experimental studies are still required to understand the exact route by which polymyxin-B induces *P. falciparum* cell death.

5.2. Limitations to the experimental approach and how they could be overcome for future projects

The current study demonstrated that LPS-free PfHsp70-1 does not induce immune cells to produce pro-inflammatory cytokine expression. However, the current study did not cater for the detection of immunosuppressive cytokines such as IL10. The next study should, therefore, use a wider range of detection assay. *In vivo,* experiments to understand the effects of PfHsp70-1 on the immune system the immunomodulatory effects of LPS-PfHsp70-1. Furthermore *In vivo* studies are still required to investigate optimal polymyxin-B dosage treatments with maximal efficacy and low toxicity levels.

The current study could not fully identify the residues utilised by PfHsp70-1 to interact with polymyxin-B. Hence, to identify the exact domain and residues involved in this interaction, SPR imaging analysis should be conducted with truncated forms of PfHsp70-1. Although, polymyxin-

B exhibited direct inhibition on *P. falciparum* cultures, it has not been shown if it can be an alternative to alleviate the current drug resistance. It is thus essential to conduct more assays with polymyxin-B on drug resistant parasite strains. In this study, the modulation of polymyxin-B on PfHsp70-1 chaperone activity was observed using *in vitro* assays and can potentially be related to the desired *in vivo* effects, however, is not clear. It is thus essential that *in vitro* work done from this study be followed by *in vivo* experiments to understand the effects of metabolism and further confirm the use of polymyxin-B as a potential antimalarial therapeutic drug.

REFERENCES

Acharya, P., Kumar, R., and Tatu, U. (2007). Chaperoning a cellular upheaval in malaria: heat shock proteins in *Plasmodium falciparum*. *Mol. Biochem. Parasitol*. **153**: 85–94.

Adams, S., Brown, H. and Turner, G. (2002). Breaking down the blood-brain barrier: signaling a path to cerebral malaria? *Trends Parasitol*. **18** (8): 3600-3606.

Akira, S., Uematsu,S. and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*. **124** (4): 783-801.

Ambade, A., Catalano, D., Lim, A. and Mandrekar, P. (2012). Inhibition of heat shock protein (molecular weight 90 kDa) attenuates proinflammatory cytokines and prevents lipopolysaccharide-induced liver injury in mice. *Hepatology*. **55**:1585–1595.

Anand, P.K., Anand, E., Bleck, C.K.E., Anes, E., and Griffiths, G. (2010). Exosomal Hsp70 induces a proinflammatory response to foreign particles including Mycobacteria. *PLoS ONE*. **5**(4): e10136.

Aosai, F., Rodriguez-Pena, M.S., Mun, H.S., Fang. H., Mitsunaga, T., Norose, K., Kang, H.K., Bae, Y.S., Yano, A. (2006). *Toxoplasma gondii*-derived heat shock protein 70 stimulates maturation of murine bone marrow-derived dendritic cells via Toll-like receptor 4. *Cell Stress & Chaperones.* **11** (1): 13-22.

Arnold, K., Bordoli, L., Kopp, J. and Schwede, T. (2006). The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics*. **22:** 195-201.

Asea, A. (2003). Chaperokine-induced signal transduction pathways. *Exerc. Immunol. Rev*. **25**-33.

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K. (2002). Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J. Biol. Chem*. **277**:15028-15034.

Aurrecoechea, C., Brestelli, J., Brunk, B.P., Dommer, J., Fischer, S., Gajria, B., Gao, X., Gingle, A.,Grant, G., Harb, O.S., Heiges, M., Innamorato, F., Iodice, J., Kissinger, J.C., Kraemer, E., Li, W., Miller, J.A., Nayak, V., Pennington, C., Pinney, D.F., Roos, D.S., Ross, C., Stoeckert, C.J. Jr., Treatman, C. and Wang, H. (2009). PlasmoDB: a functional genomic database for malaria parasites. *Nucleic Acids Research*. **37**: D539–D543.

Banerjee, T., Singh, R.R., Gupta, S., Surolia, A. and Surolia, N. (2012). 15-Deoxyspergualin hinders physical interaction between basic residues of transit peptide in PfENR and Hsp70-1. *IUBMB Life*. **64**: 99-107.

Basha E, Lee GJ, Demeler B, Vierling E (2004) Chaperone activity of cytosolic small heat shock proteins from wheat. Eur J Biochem 271: 1426–1436.

Beliakoff, J. and Whitesell, L. (2004). Hsp90: an emerging target for breast cancer therapy. *Anticancer Drugs*. **15** (7): 651-662.

Bell, S.L., Chiang, A.N., and Brodsky, J. L. (2011). Expression of a malarial Hsp70 improves defects in chaperone-dependent activities in *ssa1* mutant yeast. *PLoS ONE*. **6**: (5):e20047.

Bendz, H., Marincek, B.C., Momburg, F., Ellwart, J.W., Issels, R.D., Nelson, P.J. and Noessner, E. (2008). Calcium signaling in dendritic cells by human or mycobacterial Hsp70 is caused by contamination and is not required for Hsp70-mediated enhancement of cross-presentation. *J. Biol. Chem.* **283** (39): 26477-26483.

Bettiol E1, Carapau D, Galan-Rodriguez C, Ocaña-Morgner C, Rodriguez A. (2010). Dual effect of *Plasmodium*-infected erythrocytes on dendritic cell maturation. *Malar. J.* **9**:64.

Bhatnagar, S., Shinagawa, K., Castellino, F.J. and Schorey, J.S. (2007). Exosomes released from macrophages infected with intracellular pathogens stimulate a pro-inflammatory response *in vitro* and *in vivo*. *Blood*. **110**: 3234–3244.

Bhattacharjee, S., Stahelin, R.V., Speicher, K.D., Speicher, D.W. and Haldar, K. (2012). Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell. *Cell* **148**: 201–212.

Bhattacharjee, S., van Ooij, C., Balu, B., Adams, J.H. and Haldar, K. (2008). Maurer‟s clefts of *Plasmodium falciparum* are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. *Blood*. **111**: 2418–2426.

Bianchi, M. E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukocyte Biol*. **81**: 1–5.

Birchler, T., Seib, R, Büchner, K., Loeliger, S., Seger, R., Hossle J.P., Aguzzi, A. and Lauener, R.P. (2001). Human toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defens in response to bacterial lipoprotein. *European Journal of Immunology*. **31** (11): 3131–3137.

Blatch, G.L. and Pryzyborski, J.M. (2011). Protein biochemistry: Don't forget the cell biology. *Biochimica et Biophysica Acta* **1814** (3): 456.

Botha, M., Pesce, E.R. and Blatch, G.L. (2007). The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: regulating chaperone power in the parasite and the host. *J. Biochem. Cell Biol*. **39**: 1781- 1803.

Brinker, A., Scheufler, C., von der Mülbe, F., Fleckenstein, B., Herrmann, C., Jung, G., Moarefi, I., and Hartl, F. U. (2002). Ligand discrimination by TPR domains. Relevance and selectivity of EEVD- recognition in Hsp70-Hop-Hsp90 complexes. *J. Biol. Chem.* **277**: 1926519275.

Brodsky, J.L. and Chiosis, G. (2006). Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. *Curr. Trop. Med. Chem.* **6**: 1215–1225.

Buchberger, A., Theyssen, H., Schroder, H., McCarty, J.S., VIrgallita, G., Milkereit, P., Reinstein, J and Bukau, B. (1995). Nucleotide-induced conformational changes in the ATPase and substrate binding domains of the DnaK chaperone provide evidence for interdomain communication. *J. Biol Chem*, **270**: 16903-16910.

Bukau, B., Weissman, J., and Horwich, A. (2006). Molecular Chaperones and Protein Quality Control. *Cell*. **125**: 443-451.

Buttenschoen, K., Radermacher, P. and Bracht, H. (2010). Endotoxin elimination in sepsis: physiology and therapeutic application. *Langenbecks Arch. Surg*. **395**: 597–605.

Calderwood, S.K., Mambula, S.S. and Gray, P.J. (2007). Extracellular heat shock proteins in cell signaling and immunity. *Ann NY Acad Sci.* **1113**: 28–39.

Campos, F.M.F., Franklin, B.S., Teixeira-Carvalho, A., Filho, A.L.S., de Paula, S., Fontes, C.J., Brito, C.F. and Carvalho, L.H. (2010). Augmented plasma microparticles during acute *Plasmodium vivax* infection. *Malar. J*. **9**:327.

Caplan, A.J. (2003). What is a co-chaperone? *Cell Stress Chaperon*. **8**: 105-107.

Caraballo, H. (2014). Emergency Department Management of Mosquito-Borne Illness: Malaria, Dengue and West Nile Virus. *Emergency Medicine Practice*. **16** (5): 1-23.

Carabarin-Lima, A., González-Vázquez, M.C., Baylon-Pacheco, L., Tsutsumi, V., Talamás-Rohana, P., Rosales-Encina, J.S. (2011). Immunization with the recombinant surface protein rTcSP2 alone or fused to the CHP or ATPase subdomain of TcHSP70 induces protection against acute Trypanosoma cruzi infection. *J. Vaccin.* **1**: 110.

Cardoso, L.S., Araujo, M.I., Goes, A.M., Pacifico, L.G., Oliveira, R.R. and Oliveira, S.C. (2007). Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microb. Cell Fact*. **6**:1–6.

Cham, G.K., Turner, L., Lusingu, J., Vestergaard. L., Mmbando. B.P., Kurtis, J.D., Jensen, A.T., Salanti, A., Lavstsen, T. and Theander, T.G. (2009). Sequential, ordered acquisition of antibodies to *Plasmodium falciparum* erythrocyte membrane protein 1 domains. *J. Immunol*. **183**:3356-3363.

Chamberlain, L.H. and Burgoyne, R.D. (1997). Activation of the ATPase activity of heat-shock proteins Hsc70/Hsp70 by cysteine-string protein. *Biochem. J.* **322**: 853-858.

Chase, M.A., Wheeler, D.S., Lierl, K.M., Hughes, V.S., Wong, H.R. and Page, K. (2007). Hsp72 induces inflammation and regulates cytokine production in airway epithelium through a TLR4 and NF kappa-B dependent mechanism. *J Immunol.* **179** (9):6318–6324.

Cheetham, M.E. and Caplan, A.J. (1998). Structure, function and evolution of DnaJ: Conservation and adaptation of chaperone function. *Cell Stress and Chaperones*. **3**: 28-36.

Chen, Q., Schlichtherle, M. and Wahlgren, M. (2000). Molecular aspects of severe malaria. *Clin. Microbiol Rev.* **13** (3): 439–450.

Chiang, A.N., Valderramos, J.C., Balachandran, R., Chovatiya, R.J., Mead, B.P., Schneider, C., Bell, S.L., Klein, M.G., Huryn, D.M., Chen, X.S., Day, B.W., Fidock, D.A., Wipf, P. and Brodsky, J.L. (2009). Select pyrimidinones inhibit the propagation of the malarial parasite, *Plasmodium falciparum*. *Bioorganic and Medicinal Chemistry*. **17**: 1527-1533.

Chicz, R.M., Urban, R.G., Lane, W.S., Gorga, J.C., Stern, L.J., Vignali, D.A. and Strominger, J.L. (1992). Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC related molecules and are heterogeneous in size. *Nature*. **358**: 764–768.

Cockburn, I.L., Pesce, E.R., Pryzborski, J.M., Davies-Coleman, M.T., Clark, P.G., Keyzers, R.A., Stephens, L.L., and Blatch, G.L. (2011). Screening for small molecule modulators of Hsp70 chaperone activity using protein aggregation suppression assays: inhibition of the plasmodial chaperone PfHsp70-1. *Biol Chem.* **392**: 431-8.

Cyrklaff, M., Sanchez, C.P., Frischknecht, F. and Lanzer, M. (2012). Host actin remodeling and protection from malaria by hemoglobinopathies. Trends in Parasitology. 28, 479-485.

Dahl, E.L., Shock, J.L., Shenai, B.R., Gut, J., DeRisi, J.L. and Rosenthal, P.J. (2006). Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy*. **50**: 3124.

Daily, J.P., Scanfeld, D., Pochet, N., Le Roch, K., Plouffe, D., Kamal M., Sarr, O., Mboup, S., Ndir, O., Wypij, D., Levasseur, K., Thomas, E., Tamayo, P., Dong, C., Zhou, Y., Lander, E.S., Ndiaye, D., Wirth, D., Winzeler, E.A., Mesirov, J.P. and Regev, A. (2007). Distinct physiological states of *Plasmodium falciparum* in malaria-infected patients. *Nature* **450**: 1091–1095.

de-Koning-Ward, T.F., Gilson, P.R., Boddey, J.A., Rug, M., Smith, B.J., Papenfuss, A.T., Sanders, P.R., Lundie, R.J., Maier, A.G., Cowman, A.F. and Crabb, B.S. (2009). A newly discovered protein export machine in malaria parasites. *Nature*. **459**: 945–949.

DeLano, W.L. (2002). The PyMOL Molecular Graphics System (2002). DeLano Scientific, San Carlos,CA, USA. http://www.PyMOL.org.PyMOL

Demand, J., Lüders, J. and Hörfeld, J. (1998). The carboxy-terminal domain of Hsc70 provides binding sites for a distinct set of chaperone cofactors. *Mol. Cell. Biol.* 18: 2023–2028.

den Engelsman, J., Garidel, P., Smulders, R., Koll, H., Smith, B., Bassarab, S., Seidl, A., Hainzl, O. and Jiskoot, W. (2011). Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. *Pharm. Res* **28**: 920–933.

Edkins, A L. and Boshoff, A. (2014). General Structural and Functional Features of Molecular Chaperones. *Heat Shock Proteins of Malaria.* 5-46.

Eksi, S. and Williamson, K.C. (2011). Protein targeting to the parasitophorous vacuole membrane of *Plasmodium falciparum*. *Eukaryotic Cell.* **10**: 744–752.

Engelbrecht, D., Durand, P.M. and Coetzer, T.L. (2012). On programmed cell death *in Plasmodium falciparum*: Status Quo. *Journal of Tropical Medicine*, 646534.

Evans, C.G., Chang, L. and Gestwicki, J.E. (2010). Heat shock protein 70 (Hsp70) as an emerging drug target. *Journal of Medicinal Chemistry*. **53**: 4585–4602.

Ellis, R.J. (1987). Proteins as molecular chaperones. *Nature* **328**: 378-379.

Fevrier, B. and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr Opin Cell Biol.* **16**: 415–421.

Fiske, M.J., Fredenburg, R.A., VanDerMeid, K.R., McMichael, J.C. and Arumugham. R. (2001). Method for reducing endotoxin in Moraxella catarrhalis UspA2 protein preparations. *J. Chromatogr. B.* **753**: 269–278

Flaherty, K. M., DeLuca-Flaherty, C., McKay, D. B. (1990). Three-dimensional structure of the ATPase fragment of a 70-K heat shock cognate protein. *Nat.* **346**: 623-628.

Fletcher, J.R. and Ramwell, P.W. (1980). The effects of prostacyclin (PG12) on endotoxin shock and endotoxin-induced platelet aggregation in dogs. *Circ. Shock*. **7**: 299–308.

Fujioka, H., and Aikawa, M. (2002). Structural and life cycle. In: Malaria and parasite and disease, (eds). *Troye-Blomberg, M., and Perlmann, P*. 1-26.

Fung, K.L., Hilgenberg, K., Wang, N.M. and Chirico, W.J. (1996). Conformations of the nucleotide and polypeptide binding domains of a cytosolic Hsp70 molecular chaperone are coupled. *The J. of Biol. Chem.* 271, 21559-21565.

Gao, B. and Tsan, M.F. (2003). Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J. Biol. Chem*. **278** (1): 174-179.

Garimella, R., Liu, X., Qiao, W., Liang, X., Zuiderweg, E.R.P., Riley, M.I. and van Doren, S.R. (2006). Hsc70 contacts helix III of the J domain from polyomavirus T antigens: Addressing a dilemma in the chaperone hypothesis of how they release E2F from pRb. *Biochemistry*. **45**: 6917-6929.

Gearing, A.J.H. (2007). Targeting toll-like receptors for drug development: a summary of commercial approaches. *Immunology and Cell Biology*. **85**: 490–494

Gilman A, Goodman LS, Hardman JG, Limbird LE (2001). Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill

Gitau, G.W., Mandal, P., Blatch, G.L., Przyborski, J., and Shonhai, A. (2012). Characterisation of the *Plasmodium falciparum* Hsp70-Hsp90 organising protein (PfHop). *Cell Stress Chaperon.* **17**: 191–202.

Góes, L.C., Miranda, E.A. and Bueno, S.M.A. (2010). Interaction of histidine-tagged human proinsulin with immobilized nickel ion: effect of chelating ligand and thermodynamics analysis. *Colloids Surf A: Physicochemical and Engineering aspects*. **369** (1): 176–185.

Goloubinoff, P., Mogk, A., Peres Ben Zvi, A., Tomoyasu, T. and Bukau, B. (1999). Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proc Natl Acad Sci USA.* **96**: 13732–13737.

Gopal, J.G. and Kumar, A. (2013). Strategies for the production of recombinant protein in *Escherichia coli*. *Protein J*. **32** (6): 419-425.

Graham, F.L., Smiley, J. Russell, W.C. and Nairn. R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol*. **36:** 59-74.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, J.S. (1982). Analysis of nitrate, nitrite and nitrate in biological fluids. *Anal. Biochem.* **126**: 131–138.

Griffith, J.W., Sun, T., Mcintosh, M.T. and Bucala, R. (2009). Pure Hemozoin is inflammatory *in vivo* and activates the NALP3 inflammasome via release of uric acid. *J. Immunol.* **183**: 5208–5220.

Grover, M., Chaubey, S., Ranade, S. and Tatu, U. (2013). Identification of an exported heat shock protein 70 in *Plasmodium falciparum*. *Parasite*. **20**: 2-9.

Guirgis, B.S.S., Sá e Cunha, C., Gomes, I., Cavadas, M., Silva, I., Doria, G., Blatch, G.L., Baptista, P.V., Pereira, E., Azzazy, H.M., Mota, M.M., Prudêncio, M. and Franco., R. (2012). Gold nanoparticle-based fluorescence immunoassay for malaria antigen detection. *Anal Bioanal Chem*. **402**:1019–1027.

Habich, C., Kempe, K., van Der Zee, R., Rumenapf, R., Akiyama, H., Kolb, H. and Burkart, V. (2005) Heat shock protein 60: specific binding of lipopolysaccharide. *J. Immunol*. **174**: 1298–1305.

Hainzl, O., Lapina M.C., Buchner J. and Richter K. (2009). The charged linker region is an important regulator of Hsp90 function. *J. Biol. Chem.* **284**: 22559–22567.

Han, W. and Christen, P. (2001). Mutations in the interdomain linker region of DnaK abolish the chaperone action of the DnaK/DnaJ/GrpE system. *FEBS Letters*. **497**: 55-58

Hartl, F.U. and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Sci.* **295**: 1852–1858.

Hartl, F.U. and Hayer-Hartl, M. (2009). Converging concepts of protein folding *in vitro* and *in vivo*. *Nat Struct Mol Biol.* **16**: 574–581.

Hartl, F.U., Bracher, A. and Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*. **475** (7356): 324-332.

Heine, H. (2011) TLRs, NLRs and RLRs: Innate sensors and their impact on allergic diseases - A current view. *Immunology letters*. **139** (1-2): 14-24.

Heine, H., Müller-Loennies, S., Brade, L., Lindner, B. and Brad, H. (2003). Endotoxic activity and chemical structure of lipopolysaccharides from Chlamydia trachomatis serotypes E and L2 and Chlamydophila psittaci 6BC. *European Journal of Biochemistry*. **270** (3): 440–450.

Heiny, S.R., Spork, S. and Przyborski J.M. (2012). The apicoplast of the human malaria parasite *P. falciparum*. *Journal of Endocytobiosis and Cell Research*. **23**: 91-95.

Hennessy, F., Nicoll, W.S., Zimmermann, R., Cheetham, M.E. and Blatch, G.L. (2005). Not all J domains are created equal: Implications for the specificity of Hsp40-Hsp70 interactions. *Protein Sci*. **14**: 1697–1709.

Hermeling, S., Aranha, L., Damen, J.M., Slijper, M., Schellekens, H., Crommelin, D. J. and Jiskoot, W. (2005). Structural characterization and immunogenicity in wild-type and immune tolerant mice of degraded recombinant human interferon α2b. *Pharm. Res*. **22**: 1997–2006.

Hisaeda, H., Yasutomo, K. and Himeno, K. (2005). Malaria: immune evasion by parasites. *Int J Biochem Cell Biol*. **37** (4): 700-706.

Houry, W.A., Frishman, D., Eckerskorn, C., Lottspeich, F. and Hartl, F.U. (1999). Identification of in vivo substrates of the chaperonin GroEL. *Nature*. **402**: 147-154.

Hwang, I.Y., Quan, J.H., Ahn, M.H., Ahmed, H.A., Cha, G.H., Shin, D.W. and Lee, Y.H. (2010). *Toxoplasma gondii* infection inhibits the mitochondrial apoptosis through induction of Bcl-2 and HSP70. *Parasitol Res*. **107** (6): 1313-13121.

Janouškovec, J., Horák, A., Oborník, M., Lukeš, J. and Keeling P. J. (2010). A common red algal origin of the apicomplexan, dinoflagellate, and heterokont plastids. *Proc Natl Acad Sci.* **107**: 10949–10954.

Jiang, J., Maes, E.G., Taylor, A.B., Wang, L., Hinck, A.P., Lafer, E.M. and Sousa, R. (2007). Structural basis of the J cochaperone binding and regulation of Hsp70. *Mol. Cell*. **28**: 422-433.

Joubert, M.K., Hokom, M., Eakin, C., Zhou, L., Deshpande, M., Baker, M.P., Goletz, T.J., Kerwin, B.A., Chirmule, N., Narhi, L.O. and Jawa, V. (2012). Highly aggregated antibody therapeutics can enhance the in vitro innate and late-stage T-cell immune responses. *J Biol Chem*. **287** (30): 25266-25279.

Kabani, M. and Martineau, C.N. (2008). Multiple hsp70 isoforms in the eukaryotic cytosol: mere redundancy or functional specificity? *Curr Genomics*. **9** (5): 338-348.

Kang, H.K., Lee, H.Y., Lee, Y.N. Jo EJ, Kim, J.I., Aosai, F., Yano, A., Kwak, J.Y. and Bae Y.S. (2004). *Toxoplasma gondii* derived heat shock protein 70 stimulates the maturation of human monocyte-derived dendritic cells. *Biochem. Biophys. Res. Commun*. **322**: 899-914.

Kampinga, H.H. and Craig, E.A. (2010). The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews Molecular Cell Biology*. **11**: 579–592.

Kampinga, H.H., Hageman, J., Vos, M.J., Kubota, H., Tanguay, R.M., Bruford, E.A., Cheetham, M.E., Chen. B. and Hightower, L.E. (2009). Guidelines for the nomenclature of the human heat shock proteins. *Cell Stress Chaperones*. **14**: 105–111.

Kampinga, H.H., Kanon, B., Salomons, F.A., Kabakov, A.E. & Patterson, C. (2003). Overexpression of the cochaperone CHIP enhances Hsp70-dependent folding activity in mammalian cells. *Mol. Cell. Biol*. 23: 4948–4958.

Kappes, B., Suetterlin, B. W., Hofer Warbinek, R., Humar, R., and Franklin, R. M. (1993). Two major phospho proteins of *Plasmodium falciparum* are heat shock proteins. *Mol. Biochem. Parasitol*. **59**: 83–94.

Kaufmann, S.H.E. (1990). Heat shock proteins and the immune response. *Immunology Today*; **11**:129–136.

Kawai, T. and Akira, S. (2007). TLR signaling. *Semin Immunol*. **19** (1): 24-32.

Khazen, W., M'bika, J.P., Tomkiewicz, C., Benelli, C., Chany, C., Achour, A. and Forest, C. (2005). Expression of macrophage-selective markers in human and rodent adipocytes. *FEBS Lett*. **579** (25): 5631–5634.

Kim, C.W., Christopher, C.W. and Rha, C.K. (1996). Removal of endotoxins and nucleic acids using submicro-sized polymeric particle. *J Microbiol Biotech.* **6**: 189-193.

Külzer, S., Charnaud, S., Dagan, T., Riedel, J., Mandal, P., Pesce, E.R., Blatch, G.L., Crabb, B.S., Gilson, P.R., and Przyborski, J.M. (2012). *Plasmodium falciparum*-encoded exported Hsp70/Hsp40 chaperone/co-chaperone complexes within the host erythrocyte. *Cell Microbiol.* **14**: 1784–1795.

Külzer, S., Rug, M., Brinkmann, K., Cannon, P., Cowman, A., Lingelbach, K., Gregory L. Blatch, G., Maier, A. G., and Przyborski, J.M. (2010). Parasite-encoded Hsp40 proteins define novel mobile structures in the cytosol of the *P. falciparum*-infected erythrocyte. *Cell Microbiol.* **12:** 1398– 1420.

Kumar, N., Koski, G., Harada, M., Aikawa, M. and Zheng, H. (1991). Induction and localisation of *Plasmodium falciparum* stress proteins related to the heat shock protein 70 family. *Mol Biochem Parasitol*. **48**: 47–58.

Kumar, N., Zhao, Y., Graves, P., Perez Folgar, J., Maloy, L., and Zheng, H. (1990). Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. *Infect Immun.* **58**: 1408-1414.

Landman, D., Georgescu, C., Martin, D. A. and Quale, J. (2008) Polymyxins revisited. *Clin. Microbiol. Rev.* **21**: 449–465.

Lanzer, M., Wickert, H., Krohne, G., Vincensini, L. and Braun-Breton, C. (2006). Maurer's clefts: A novel multi-functional organelle in the cytoplasm of *Plasmodium falciparum*-infected erythrocytes. *Int. J. for Parasit.* **36**: 23-36.

Li, J., Qian, X. and Sha, B. (2009). Heat shock protein 40: structural studies and their functional implications. *Protein Pept. Lett.* **16**: 606–612

Li, J., Shang, G., You, M., Peng, S., Wang, Z., Wu, H. and Chen, G.Q. (2011). Endotoxin Removing Method Based on Lipopolysaccharide Binding Protein and Polyhydroxyalkanoate Binding Protein. *PhaP Biomacromolecules*. **12**: 602–608.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991). *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. *Proc Natl Acad Sci U.S.A.* **88**: 2874–2878.

Lindner, J., Meissner, K.A., Schettert, I. and Wrenger, C. (2013). Trafficked Proteins—Druggable in *Plasmodium falciparum*?. *Intl. J Cell Biol.* Article ID 435981.

Lingelbach, K., and Joiner, K.A. (1998). The parasitophorous vacuole membrane surrounding *Plasmodium* and Toxoplasma: an unusual compartment in infected cells. *J Cell Sci.* **111**: 1467–1475.

Litman, G.W., Cannon, J.P. and Dishaw, L.J. (2005). Reconstructing immune phylogeny: new perspectives. *Nature Reviews. Immunology.* **5** (11): 866–79.

Liu, K. and Houry, W.A. (2014). Chaperones and proteases of *Plasmodium falciparum*. *Heat Shock Proteins of Malaria*. 161-188.

Lu, Z. and Cyr, D.M. (1998). Protein folding activity of Hsp70 is modified differentially by the Hsp40 co-chaperones Sis1 and Ydj1. *J. Biolog. Chem.* **273**: 27824-27880.

Luong, M., Zhang, Y., Chamberlain, T., Zhou, T., Wright, J.F., Dower, K. and Hall, J.P. (2012) Stimulation of TLR4 by recombinant HSP70 requires structural integrity of the HSP70 protein itself. *J Inflamm (Lond)*. **9**:11.

Luthuli, S.D., Chili, M.M., Revaprasadu, N. and Shonhai, A. (2013). Cysteine-capped gold nanoparticles suppress aggregation of proteins exposed to heat stress. *IUBMB Life*. **65**: 454–461

Magalhaes, P.O., Lopes, A.M., Mazzola, P.G., Rangel-Yagui, C., Penna, T.C. and Pessoa, A. Jr. (2007). Methods of endotoxin removal from biological preparations. *J Pharm Sci.* **10** (3):388-404.

Maier, A.G., Cooke, B.M., Cowman, A.F., and Tilley, L. (2009). Malaria parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol. 7: 341–354.

Makumire, S., Chakravadhanula, V.S., Köllisch, G., Redel, E. and Shonhai, A. (2014). Immunomodulatory activity of zinc peroxide (ZnO_2) and titanium dioxide (TiO_2) nanoparticles and their effects on DNA and protein integrity. *Toxicol. Lett*. **227** (1):56-64.

Mamat U, Woodard RW, Wilke K, Souvignier C, Mead D, Steinmetz E, Terry K, Kovacich C, Zegers A and Knox C. 2013. Endotoxin-free protein production—ClearColi™ technology. *Nature Methods*. 10-11

Marenchino, M., Armbruster, D.W., and Hennig, M. (2009). Rapid and efficient purification of RNA-binding proteins: application to HIV-1 Rev. *Protein Expr Purif.* **63**:112-119.

Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D., and Wishnok, J.S. (1988) Macrophage oxidation of L-arginine to nitrite and nitrate:nitric oxide is an intermediate. *Biochemistry* **27:** 8706–8711.

Marsh, K. and Kinyanjui, S. (2006). Immune effector mechanisms in malaria. *Parasite Immunol.* **28** (1–2): 51–6010.

Matambo, T.S., Odununga, O.O., Boshoff, A. and Blatch, G.L. (2004). Overproduction purification, and characterization of the *Plasmodium falciparum* heat shock protein 70. *Protein Expr. Purif.* **33:** 214– 222.

Matambo, T.S., Odununga, O.O., Boshoff, A., and Blatch, G.L. 2004. Overproduction, purification, and characterization of the Plasmodium falciparum heat shock protein 70.Prot. Expr. Purif.33:214–222.

Mayer M (2013) Hsp70 chaperone dynamics and molecular mechanism. Trends in Biochemical sciences **38** (10): 507-513.

Mayer, M., Rudiger, S. and Bukau, B. (2000b). Molecular basis for interactions of the DnaK chaperone with substrates. *Biol Chem*. **381**: 877–885.

Mbengue, A., Berry, L. and Braun-Breto, C. (2014). Establishment of *Plasmodium falciparum* extracellular compartments in its host erythrocyte. *Heat Shock Proteins of Malaria*. 133-160.

Mbengue, A., Yam, X.Y., and Braun-Breton, C. (2012). Human erythrocyte remodelling during *Plasmodium falciparum* malaria parasite growth and egress. *J Haematol.* **157**: 171-9.

Midorikawa, Y. and Haque, Q.M. (1997). 15-Deoxyspergualin, an immunosuppressive agent, used in organ transplantation showed suppressive effects on malarial parasites. *Chemotherapy.* **43**: 31-35.

Misra, G. and Ramachandran, R. (2009). Hsp70-1 from *Plasmodium falciparum*: protein stability, domain analysis and chaperone activity. *Biophys Chem.* **142**: 55–64.

Mun, H.S., Aosai, F., Norose, K., Chen, M., Hata, H.,Tagawa, Y., Iwakura, Y., Byun, D. and Yano, A. (2000). Toxoplasma gondii Hsp70 as a danger signal in *Toxoplasma gondii* infected mice. *Cell Stress Chaperones*. **54**: 328–335.

Muralidharan, V., Oksman, A., Pal, P., Lindquist, S. and Goldberg, D.E. (2012). *Plasmodium falciparum* heat shock protein 110 stabilizes the asparagine repeat-rich parasite proteome during malarial fevers. *Nature Communications*. **3**: 1310.

Nadler, S.G., Dischino, D.D., Malacko, A.R., Cleaveland, J.S., Fujihara, S.M. and Marquardt, H. (1998). Identification of a binding site on Hsc70 for the immunosuppressant 15-deoxyspergualin. *Biochmical and Biophysical Research Communications.* **253**: 176-180.

Njunge, J. M., Ludewig, M. H., Boshoff, A., Pesce, E.R., & Blatch, G. L. (2013). Hsp70s and J proteins of Plasmodium parasites infecting rodents and primates: structure, function, clinical relevance, and drug targets. *Current Pharmaceutical Design*. **19** (3): 387-403.

Nyalwidhe, J. and Lingelbach, K. (2006). Proteases and chaperones are the most abundant proteins in the parasitophorous vacuole of *Plasmodium falciparum*-infected erythrocytes. *Proteomics.* **6**: 1563-1573.

Opekarova and Tanner (2003). Specific lipid requirements of membrane proteins--a putative bottleneck in heterologous expression. *Biochim Biophys Acta*. **1610** (1):11-22.

Osterloh, A., Kalinke, U., Weiss, S., Fleischer, B. and Breloer, M. (2007). Synergistic and differential modulation of immune responses by Hsp60 and lipopolysaccharide. *J Biol Chem*. **282** (7): 4669-4680.

Osterloh, A., Veit, A., Gessner, A., Fleischer, B. and Breloer, M. (2008). Hsp60-mediated T cell stimulation is independent of TLR4 and IL-12. *Int Immunol*. **20** (3):433-443.

Ouderkirk, J.P., Nord, J.A., Turett, G.S. and Kislak, J.W. (2003). Polymyxin-B nephrotoxicity and efficacy against nosocomial infections caused by multidrug resistant gram-negative bacteria. *Antimicrobial agents Chemother*. **47** (8): 2659-2662.

Pallavi, R., Acharya, P., Chandran, S., Daily, J.P., and Tatu, U. (2010). Chaperone expression profiles correlate with distinct physiological states of *Plasmodium falciparum* in malaria patients. *Malaria J*. **9**: 236.

Pallavi, R., Roy, N., Nageshan, R.K., Talukdar, P., Pavithra, S.R., Reddy, R., Venketesh, S., Kumar, R., Gupta, A.K., Singh, R.K., Yadav, S.C. and Tatu, U. (2010). Heat Shock Protein 90 as a drug target against protozoan infections. *J. Biol. Chem.* **285**: 37964–37975.

Park, A.Y. and Scott, P. (2001). IL-12: keeping cell-mediated immunity alive. *Scand. J. Immunol*. **53** (6): 529–532.

Park, B.S. and Lee, J. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & Molecular Medicine.* **45**:e66.

Pavithra, S. R., Banumathy, G., Joy, O., Singh, V. and Tatu, U. (2004). Recurrent fever promotes *Plasmodium falciparum* development in human erythrocytes. *J Biol Chem.* **279**: 46692– 46699.

Pavithra, S.R., Kumar, R., and Tatu, U. (2007). Systems analysis of chaperone networks in the malarial parasite *Plasmodium falciparum*. *PLoS Comput Biol*. **3**: 1701–1715.

Perkins, M.D. and Bell, D.R. (2008). Working without a blindfold: The critical role of diagnostics in malaria control. *Malaria Journal* **1** (Suppl 1): S5.

Pesce, E. R., Acharya, P., Tatu, U., Nicoll, W.S., Shonhai, A., Hoppe, H.C. and Blatch, G.L. (2008). The Plasmodium falciparum hzeat shock protein 40, Pfj4, associates with heat shock protein 70 and shows similar heat induction and localisation patterns. *J Biochem Cell Biol*. **40**: 2914–2926.

Petsch, D. and Anspach F.B. (2000). Endotoxin removal from protein solutions. *J. Biotechnol*. **76**: 97–119.

Planelles L, Thomas M, Pulgar M, Marañón C, Grabbe S, López MC (2002) Trypanosoma cruzi heat-shock protein-70 kDa,alone or fused to the parasite KMP11 antigen, induces functional maturation of murine dendritic cells. *Immunol Cell Biol*. **80** (3):241-247.

Polpanich, D., Tangboriboonrat, P., Elaissari, A. and Udomsangpetch, R. (2007). Detection of malaria infection via latex agglutination assay. *Anal. Chem.* **79**: 4690–4695.

Poulaki, V., Iliaki, E., Mitsiades, N., Mitsiades, C.S., Paulus, Y.N., Bula, D.V., Gragoudas, E.S. and Miller J.W. (2007). Inhibition of Hsp90 attenuates inflammation in endotoxin-induced uveitis. *FASEB J*. **21** (9): 2113-2123.

Pratt, W.B. and Toft, D.O. (2003). Regulation of signalling protein function and trafficking by the Hsp90/hsp70-based chaperone machinery. *Experimental Biology and Medicine.* **228**: 111-133.

Qazi, K.R., Qazi, M.R., Julián, E., Singh, M., Valugerdi, M.A. and Fernández, C. (2005). Exposure to Mycobacteria primes the immune system for evolutionarily diverse heat shock proteins. *Infect. Immun.***73** (11)7687-7696.

Qazi, K.R., Wikman, N., Vasconcelos, N.M., Berzins, K., Stahl, S. and Fernandez, C. (2005). Enhancement of DNA vaccine potency by linkage of *Plasmodium falciparum* malarial antigen gene fused with a fragment of Hsp70 gene. *Vaccine*. **23:** 1114-1125.

Ralph, S. A., Van Dooren, G. G., Waller, R. F., Crawford, A. F. and Fraunholz, M., (2004). Metabolic maps and functions of the *Plasmodium falciparum* apicoplast. *Nat. Rev*. *Microbiol*. 2, 23–216.

Ramya, T.N.C., Surolia, N. and Surolia, A. (2006). 15-Deoxyspergualin modulates *Plasmodium falciparum* heat shock protein function. *Biochemical and Biophysical Research Communications*. **348:** 585-592.

Ramya, T.N.C., Surolia, N. and Surolia, A. (2007) 15-Deoxyspergualin inhibits eukaryotic protein synthesis through eIFa phosphorylation. *Biochem. J*. **401** (31): 411–420.

Ran R, Zhou G, Lu A, Zhang L, Tang Y, Rigby AC, Sharp FR. (2004) Hsp70 mutant proteins modulate additional apoptotic pathways and improve cell survival. Cell Stress & Chaperones **9** (3): 229–242.

Rathod, P.K., McErlean, T. and Lee, P.C. (1997). Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U. S. A.* **94**:9389-9393

Reichelt, P., Schwarz, C. and Donzeau, M. (2006). Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein Expr. Purif.*, **46**: 483–488.

Rigano, R., Buttari, B., Profumo, E., Ortona, E., Delunardo, F., Margutti, P., Mattei, V., Teggi, A., Sorice, M. and Siracusano, A. (2007). Echinococcus granulosus antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards a Th2 cell response. *Infect Immun*, **75**: 1667-1678.

Ritossa, F. (1962). A new puffing pattern induced by heat shock and DNP in Drosophila. *Experientia*. **18**: 571-573.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R. and Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nature Cell Biology*. **9**:1081–1088.

Rousseau, F., Schymkowitz, J. and Serrano, L. (2006). Protein aggregation and amyloidosis: confusion of the kinds? *Curr. Opin. Struct. Biol.* **16**:118–126.

Roy, N., Nageshan, R.K., Ranade, S. and Tatu, U. (2012). Heat shock protein 90 from neglected protozoan parasites. *Molecular Cell Research*. **1823**, (3): 707–711.

Rüdiger, S., Germeroth, L., Schneider-Mergener, J. and Bukau, B. (1997). Substrate specificity of the DnaK chaperone determined by screening cellulose peptide libraries. *EMBO J.* **16:** 1501-1507.

Ryder, S. P., Recht, M. I., and Williamson, J. R. (2008). Quantitative analysis of protein–RNA interactions by gel mobility shift. *Methods Mol. Biol.* **488**: 99–115.

Safeukui, I., Vatan, R., Dethoua, M., Agbo, H., Haumont, G., Moynet, D., Malvy, D., Vincendeau, P, Mossalayi, D. and Millet, P. (2008). A role of IgE and CD23/NO immune pathway in age-related resistance of Lewis rats to *Plasmodium berghei* Anka. *Microbes Infect*. **10** (12-13): 1411-1416.

Sakata, G., Yoshimura, K., Sakamoto, I., Todokoro, M. and Kunitake. M. (2011). Selective removal of endotoxin from a DNA solution by cross-linked cyclodextrin beads. *Anal. Sci*. **27**: 213–216.

Sam-Yellowe, T.Y. (2009). The role of the Maurer's clefts in protein transport in *Plasmodium falciparum*. *Trends Parasitol.* **25**: 277-284.

Sargeant, T.J., Marti, M., Caler, E., Carlton, J.M., Simpson, K., Speed, T.P. and Cowman, A.F. (2006). Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome Biology*, **7**: R12.1-R12.22.

Saridaki, T., Sanchez, C.P., Pfahler, J. and Lanzer, M. (2008). A conditional export system provides new insights into protein export in *Plasmodium falciparum*-infected erythrocytes. *Cell Microbiol*. **10** (12): 2483-2495.

Schantz-Dunn, J. and Nour N.M. (2009). Malaria and Pregnancy: A Global Health Perspective. *Rev Obstet Gynecol. Summer*. **2**(3): 186–192.

Schartz, N.E., Chaput, N., Andre, F. and Zitvogel, L. (2002). From the antigen-presenting cell to the antigen-presenting vesicle: the exosomes. *Curr Opin Mol Ther*. **4**: 372–381.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H. Hartl, F.U. and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell*. **101**: 199-210.

Schwarzer, E., Turrini, F., Ulliers, D., Giribaldi, G., Ginsburg, H. and Arese, P. (1992). Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J. Exp. Med.* **176**: 1033–1041.

Seymour, G.J. and Gemmell, E. (2001). Cytokines in periodontal disease: where to from here? *Acta Odontol Scand*. **59**: 167-173.

Sharma, V., Samant, R., Hegde, A. and Bhaja, K. (2012). Autoimmune hemolysis in malaria: a report of three cases. *J Assoc Physicians*. **60**: 129-131.

Shonhai, A., Boshoff, A. and Blatch, G.L. (2005). *Plasmodium falciparum* heat shock protein 70 is able to suppress the thermosensitivity of an Escherichia coli DnaK mutant strain. *Molecular Genetics and Genomics*. **274**: 70-78.

Shonhai, A. (2007). Molecular Characterisation of the Chaperone Properties of *Plasmodium falciparum* Heat Shock Protein 70. *PhD Thesis, Rhodes University, Grahamstown, South Africa*.

Shonhai, A., Boshoff, A. and Blatch, G.L. (2007). The structural and functionally diversity of Hsp70 proteins from *Plasmodium falciparum*. *Protein Sci*. **16**: 1803-1818.

Shonhai, A., Botha, M., de Beer, T.A.P., Boshoff, A. and Blatch, G.L. (2008). Structure-function study of *Plasmodium falciparum* heat shock protein 70 using three dimensional modeling and *in vitro* analysis. *Protein Pept. Lett*. **15**: 1117–1125.

Shonhai, A. (2010). Plasmodial heat shock proteins: targets for chemotherapy. *FEMS Disease and Pathogens*. **58**: 61–74.

Shonhai, A., Maier, A.G., Przyborski, J.M. and Blatch, G.L. (2011). Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis. *Protein Pept Lett*. **18** (2):143-57.

Shonhai, A. (2014). The role of Hsp70s in the development and pathogenicity of *Plasmodium* species. *Heat Shock Proteins of Malaria* 47-69.

Singh, S.K. (2011). Impact of product-related factors on immunogenicity of biotherapeutics. *J. Pharm. Sci.* **100:** 354–387

Šlapeta, J. and Keithly, J.S. (2004). Cryptosporidium parvum mitochondrial-type Hsp70 targets homologous and heterologous mitochondria. *Eukaryotic Cell.* **3**: 483-494.

Smith, S., Witkowski, A., and Joshi, A. K. (2003) Structural and func- tional organization of the animal fatty acid synthase. *Prog. Lipid Res.* **42**: 289–317. 20.

Sponaas, A.M., Cadman, E.T., Voisine, C., Harrison, V., Boonstra, A., O'Garra, A. and Langhorne, J. (2006). Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells, *J. Exp. Med.* **203**: 1427–1433.

Srivastava, P. K. (2008). New jobs for ancient chaperones. *Sci. Am*. **299**: 50–55

Stanway, R., Witt, T., Zobiak, B., Aepfelbacher, M. and Heussler, V. (2009). GFP-targeting allows visualization of the apicoplast throughout the life cycle of live malaria parasites. *Biol of the Cell*. **101**: 415–430.

Stephens, L.L., Shonhai, A. and Blatch, G.L. (2011). Co-expression of the Plasmodium falciparum molecular chaperone, PfHsp70, improves the heterologous production of the antimalarial drug target GTP cyclohydrolase I, PfGCHI. *Protein Express. Purif.* **77**: 159–165.

Stirling, P.C., Lundlin, V.F. and Leroux, M.R. (2003). Getting a grip on non-native proteins. *EMBO J*. **4**: 565-570.

Suh, K.N. Kain, K.C. and Keystone, J.S. 2004. Malaria. *Can. Med. Assoc. J.* **170**: 1693–1702.

Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi P, Figueiredo J-L, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ. (2009). identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* **325**: 612-616.

Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B. and Hartl, F.U. (1994). The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE. *PNAS.* **91**: 10345-10349

Takeuchi, O. and Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*. **140** (6):805-820.

Tamez, P.A.., van Ooij, C., Hiller, N.L., Linás, M., Balu, B., Adams, J.H. and Haldar, K. (2008). An Erythrocyte Vesicle Protein Exported by the Malaria Parasite Promotes Tubovesicular Lipid Import from the Host Cell Surface. . *PLOS Pathogens*.**4** (8): e1000118.

Taramelli D, Recalcati S, Basilico N, Olliaro P, Cairo G (2000) Macrophage preconditioning with synthetic malaria pigment reduces cytokine production via heme iron-dependent oxidative stress. *Lab. Invest.* **80**: 1781–1788.

Tate, C.G., Haase, J., Baker, C., Boorsma, M., Magnani, F., Vallis, Y. and Williams, D.C. (2003). Comparison of seven different heterologous protein expression systems for the production of the serotonin transporter. *Biochim Biophys Acta*. **1610** (1): 141–153.

Testori,A. Richards, J., Whitman, E., Mann, GB., Lutzky, J., Camacho, L., Parmiani, G., Tosti, G., Kirkwood, J.M., Hoos, A., Yuh, L., Gupta, R., Srivastava, P.K. and C-100-21 Study group. (2008). Phase III comparison of vitespen, an autologous tumor-derived heat shock protein gp96 peptide complex vaccine, with physician's choice of treatment for stage IV melanoma: *J. Clin. Oncol.* **26**: 955-962.

Thirumalai D., and Lorimer G.H. (2001) Chaperonin-mediated protein folding. *Annu. Rev. Biophys. Biomol. Struct*. **30**: 245–269.

Tobian, A. A. R., Canaday, D. H. and Harding, C. V. (2004). Bacterial heat shock proteins enhance class II MHC antigen processing and presentation of chaperoned peptides to CD4+ T cells. *J. Immunol*. **173**: 5130–5137.

Triantafilou M, and Triantafilou K. (2004). Heat-shock protein 70 and heat-shock protein 90 associate with Toll-like receptor 4 in response to bacterial lipopolysaccharide. *Biochem Soc Trans.* **32**: 636–639.

Tsubery, H., Ofek, I., Cohen, S. and Fridkin, M. (2000). The functional association of polymyxin-B with bacterial lipopolysaccharides is stereospecific: studies on polymyxin-B nonapeptide. *Biochemistry*. **39**: 11837-11844.

Tsutsumi S., Mollapour M., Graf C., Lee C. T., Scroggins B. T., Xu W., Haslerova L., Hessling M., Konstantinova A. A., Trepel J. B., Panaretou B., Buchner J., Mayer M. P., Prodromou C., Neckers L.(2009). Hsp90 charged-linker truncation reverses the functional consequences of weakened hydrophobic contacts in the N domain. *Nat. Struct. Mol. Biol.* **16**: 1141–1147.

Tynan, G.A., McNaughton, A., Jarnicki, A., Tsuji, T. and Lavelle, E.C. (2012). Polymyxin B inadequately quenches the effects of contaminating lipopolysaccharide on murine dendritic cells. *PLOS ONE*. **7** (5): e37261

Udono, H. and Srivastava, P.K. (1993). Heat shock protein 70-associated peptides elicit specific cancer immunity. *J. Exp. Med.* **178**: 1391–1396.

Urban B.C., Ferguson D.J., Pain A., Willcox N., Plebanski., M. Austyn J.M and Roberts D.J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells, *Nature*. **400**: 73–77.

van Eden, W., van der Zee, R. and Prakken, B. (2005). Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat. Rev. Immunol*. **5**: 318–330.

Vincensini, L., Richert, S., Blisnick, T., Van Dorsselaer, A., Leize-Wagner, E., Rabilloud, T., and Braun, B.C. (2005). Proteomic analysis identifies novel proteins of the Maurer‟s clefts, a secretory compartment delivering *Plasmodium falciparum* proteins to the surface of its host cell. *Mol. Cell Proteom.* **4**: 582–593.

Waller, R.F., Keeling, P.J., Donald, R.G.K., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A.F., Besra, G.S., Roos, D.S. and McFadden G.I. (1998). Nuclear-encoded proteins target to the plastid in *Toxoplasma gondii* and *Plasmodium falciparum. Proc. Natl. Acad. Sci*. **95**: 12352–12357.

Wang, L.Z., Wang, X.S., Li, Y.H., Bai, Z.P., Xiong, R.G., Xiong, M. and Li, G.W. (2002). *Chin. J. Inorg. Chem.* **18**: 1191–1194.

Wareham, D.W. and Bean D.C. (2006). *In vitro* activities of polymyxin-B, imipenem and rifampin against multidrug resistant *Actinetobacter baumannii*. *Antimicrobial Agents CH*. **50** (2): 825-825.

Wei, Z., Huang, W., Li, J., Hou, G., Fang, J. and Yuan, Z. (2007). Studies on endotoxin removal mechanism of adsorbents with amino acid ligands. *J. Chromatogr. B.* **852**: 288–292.

Wendling,U., Paul, L., van der Zee, R., Prakken, B., Singh, M., van Eden, W. (2000). A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. *J Immunol*; 164:2711–17.

Wheeler, D.S., Chase, M.A., Senft, A.P., Poynter, S.E., Wong, H.R. and Page, K. (2009). Extracellular Hsp72, an endogenous DAMP, is released by virally infected airway epithelial cells and activates neutrophils via Toll-like receptor (TLR)-4. *Respir Res.* **10**:31.

White, N. J. (2008). *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis*. **46**: 172–173.

Wilson, R.J., Denny, P.W., Preiser, P.R., Rangachari, K., Roberts, K., Roy, A., Whyte, A., Strath, M., Moore, D.J., Moore, P.W. and Williamson, D.H. (1996). Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum. J. Mol. Biol*. **261**: 155–172.

Wirth, D.F. (2002). The parasite genome: biological revelations. *Nature.* **419**: 495-496.

Wisniewska, M., Karlberg, T., Lehtiö, L., Johansson, I., Kotenyova, T., Moche, M. and Schüler, H. (2010). Crystal structures of the ATPase domains of four human Hsp70 isoforms: HSPA1L/Hsp70-hom, HSPA2/Hsp70-2, HSPA6/Hsp70B, and HSPA5/BiP/GRP78. *PLoS ONE* 5: e8625.

Wittung-stafshede, P., Guidry, J., Horne, B.E. and Landry, S.J. (2003). The J-Domain of Hsp40 couples ATP hydrolysis to substrate capture in Hsp70. *Biochemistry*. **42**: 4937-4944.

World Health Organization (2010). *World Malaria Report 2010*. Geneva: World Health Organization.

World Health Organization (2012). *World Malaria Report 2012*. Geneva: World Health Organization.

Wright, C.M., Chovatiya, R.J., Jameson, N.E., Turner, D.M., Zhu, G., WerneR, S., Huryn, D.M., Pipas, J.M., Day, B.W., Wipf, P. and Brodsky, J.L. (2008). Pyrimidinone-peptoid hybrid molecules with distinct effects on molecular chaperone function and cell proliferation. *Bioorg. Med. Chem.* **16**: 3291-3301.

Wykes M. Keighley, C., Pinzon-Charry, A. and Good, M.F. (2007). Dendritic cell biology during malaria. *Cell. Microbiol.* **9**: 300–305.

Yeh, E. and DeRisi, J.L. (2011). Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum. PLoS Biol.* **9** (8):e1001138.

Young, D., Lathigra, R., Hendrix, R., Sweetser, D. and. Young, R.A. (1988). Stress proteins are immune targets in leprosy and tuberculosis. *Proc. Natl. Acad. Sci.* **59**: 4267–4270.

Zhu, X., Zhao, X., Burkholder, W.F., Gragerov, A., Ogata, C.M., Gottesman, M.E. and Hendrickson, W.A. (1996). Structural analysis of substrate binding by the molecular chaperone DnaK. *Science.* **272**: 1606–1614.

Zügel, U. and Kaufmann, S.H., (1999). Immune response against heat shock proteins in infectious diseases. *Immunobiology*. **201** (1): 22-35.

APPENDIX

GENERAL EXPERIMENTAL PROCEDURES, SUPPLEMENTARY DATA AND LIST OF SPECIALISED REAGENTS

Appendix A: General experimental procedures

A. 1 Preparation of competent *E. coli* XL1Blue cells

Colony of E. *coli* XL1Blue was inoculated in 5ml 2x YT broth (1.6 g tryptone, 1.0 g yeast, 1.5 g agar, 0.5 g NaCl per 100 ml preparation in deionised water) broth and grown overnight with shaking at 37 ºC. The overnight culture was diluted 1:200 into 50ml YT broth and thereafter grown with shaking to early log phase of absorbance 0.3-0.6 measured at 600nm. The cells were harvested by centrifuging at 5,000g for 10 minutes at 4 ºC. The cells were kept on ice from this point onwards. The cells were resuspended in 10ml 0.1 M MgCl2 and left on ice for 30 minutes. The suspension was centrifuged for 10 minutes at 4000g at 4 ºC. The cells were pelleted as before and gently resuspended in 10 ml ice cold 0.1 M $CaCl_2$ and then followed by incubation on ice for 4 hours. Centrifugation was carried out at 4000g at 4 ºC for 10 minutes. The competent cells were aliquoted by adding 1 volume of sterile 30% glycerol mixing and stored at -80 ºC until use.

A. 2 Transformation of competent cells

A volume of 2 µl of plasmid DNA was added into an aliquot of 100 µl of competent cell. The cells were then incubated on ice for 30 minutes followed by heat shocking at 42°C for 45 seconds and immediately placed on ice for 10 minutes. Volume of 900 µl of 2x YT broth was added and then incubated at 37°C for 1 hour. The cells were transferred on 2x YT plates containing the desired antibiotics followed by incubation at 37°C overnight.

A. 3 Extraction of plasmid DNA

Plasmid DNA was extracted using Zyppy™ Plasmid Miniprep Kit according to supplier's protocol.

A. 4 Restriction digest of Plasmid DNA using enzymes

Plasmid DNA was digested using the desired diagnostic restriction enzymes following the method described below. The reagents were set up as follows: Sterile deionised water (16 µl), 10x restriction buffer (2 µl) and DNA (100-200 ng) 2 µl. The reaction was initiated by addition

of 2 units of restriction enzymes. The restriction was allowed to proceed for 2-3 hours at 37ºC. The reaction was stopped by addition of 4 µl of 10x DNA loading buffer (0.25% bromophenol blue and 30% glycerol). The product was then analysed by agarose gel electrophoresis as described in (Appendix A. 5).

A. 5 Agarose gel electrophoresis

To prepare 0.8% (w/v) agarose gel, the required amount of agarose was completely dissolved in 1x TAE buffer (40 mM, 20 mM acetic acid and 1 mM EDTA) by heating with frequent agitation. The agarose was then cooled to 55ºC prior to addition of ethidium bromide (0.5 µg/ml). The agarose gel was allowed to polymerise for 15-30 minutes at room temperature. The gel was placed in the electrophoresis chamber and covered with 1x TAE buffer. Volume of 4 µl of 10x DNA loading buffer (0.25% bromophenol blue + 30% glycerol) was added to 20 µl of sample followed by loading of the samples into the wells. Electrophoresis was conducted at 100 volts for 1 hour. The gel was then visualised using UV light (GeneGenius Bioimaging System (Syngene), USA).

A. 6 Extraction of DNA from agarose gel

After electrophoresis DNA was extracted from the agarose gel using the ZymocleanTM Gel DNA Recovery Kit according to the manufacturer‟s protocol. Briefly, after identification of the DNA fragment using UV light, the DNA was excised from the gel using a sterile scalpel and transferred to a 1.5 ml microcentrifuge tube and 3 volumes of ADB buffer added to each volume of agarose excised from the gel. The agarose was then incubated at 55 ºC for 10 minutes until the gel slice was completely dissolved. The melted agarose solution was transferred to a Zymo–spin column in a collection tube followed by centrifugation at ≥10 000 x g for 30–60 seconds and the flow–through was discarded. The DNA was washed twice with wash buffer and spun down at ≥ 10 000 x g for 30 seconds. The flow–through was discarded and the DNA was eluted using ≥ 6 µl sterile distilled water and centrifugation at ≥10 000 x g for 30 – 60 seconds to elute DNA.

A. 7 Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were treated by boiling in SDS sample buffer (0.25% Coomasie Brilliant blue (R250), 2% SDS, 10% glycerol (v/v), 100 mM tris, and 1% mercaptoethanol) in a ratio of 4:1 for 5 minutes at 95°C and resolved using 12% acrylamide resolving gel prepared as shown below (Table A.1). The gel is then transferred into the electrophoresis tank and electrophoresis buffer (25 mM Tris, pH 8.3 250 mM glycine and 0.1% (w/v) SDS) was added. The boiled samples were loaded in respective wells and prestained protein molecular weight markers (ThermoFisher Scientific, USA) were also loaded. The electrophoresis was performed at 150 volts for one hour using the Bio-Rad Mini protein electrophoresis system (Biorad, U.S.A).

Table A.1 Preparation of SDS-PAGE

Reagent (ml)	*12% Separating gel*	*5% Stacking gel*
30% Bis/acrylamide	2.08	0.235
10% Ammonium persulphate	0.025	0.00875
1.5 M Tris (pH 8.8)	1.25	
1.0 M Tris (pH 6.8)		0.437
10% SDS	0.05	0.0175
Distilled water	1.58	1.05
TEMED	0.0025	0.03

A. 8 Native polyacrylamide gel electrophoresis (native-PAGE)

Proteins were treated in native gel sample buffer (31.25 mM Tris-HCl (pH 6.8), 12.5% glycerol and 0.5% Bromophenol Blue) in a 4:1 ratio. The treated proteins were then resolved using 12% native acrylamide gel prepared as shown below (Table A.2). The gel was then transferred into the electrophoresis tank and native-PAGE electrophoresis buffer (25 mM Tris; 192 mM glycine, pH 8.3). The samples were loaded in respective wells and electrophoresis was performed at 100 volts for one hour. After electrophoresis the gel was stained with Coomassie-blue or used for Western blot analysis (Appendix A. 9).

Table A.2 Preparation of native PAGE

Reagents	***12% Separating Gel***	***2% Stacking gel***
30% Bis/acrylamide	4ml	0.67 ml
0.375M Tris (pH=8.8)	5.89ml	4.275 ml
10% Ammonium persulphate	100µl	100 µl
TEMED	10µl	10µl

A. 9 Western Blot

Proteins were resolved in 12% acrylamide gel as described above (Appendix A.7 and A. 8). Removal of SDS-PAGE gel from the glass plates after completion of electrophoresis process and cutting off of the stacking gel was done. The Whatman filter papers, gel, two scotchbrite fibre pads and nitrocellulose were emmersed in western transfer paper and left to equilibrate at 8ºC for 30 minutes. Preparation of the gel for transfer was done as follows: filter paper was placed on a scotch brite pad; Gel was placed on the filter paper ensuring no air bubbles are trapped; nitrocellulose was placed over the gel; another filter paper was laid on top of the nitrocellulose, followed by another scotch brite pad. The transfer of the protein on the nitrocellulose membrane was performed by running at 100 volts for 1 hour.

The membrane was removed from the sandwich and rinsed using transfer buffer as well as removal of adhering gel on the nitrocellulose membrane using a cotton swab. The blot was stained with ponceau stain to determine the success of the transfer followed by visualising the band using chemiluminescence. The membrane was blocked in 10 ml of (5% nonfat milk in TBS) for 1 hour on a rotary shaker set at 1 rpm. The membrane was washed three times in TBS-Tween for 10 minutes followed by incubation of the membrane with primary antibody for 1 hour. Unbound primary antibody were removed by washing of the membrane three times using TBS-Tween for 10 minutes each wash. The membrane was incubated with secondary antibody

for 1 hour followed by washing of the membrane 3 times using TBS-Tween. Images were acquired using X-ray film (Pierce CL-Xposure™ Film, Thermo Scientific, USA).

A. 10 Determination of protein concentration using Bradford assay

Protein concentration was determined by Bradford‟s method (Bradford, 1976). Bovine serum albumen (BSA) standards were prepared using concentration ranging from 0 to 30 µg/ml in 0.15 M NaCl. Bradfords reagent 200 µl (Sigma Aldrich, USA) was added to 10 µl of protein and the reaction incubated in the dark at room temperature for 5 minutes. Absorbance was read at 595 nm using (Das plate reader, Italy).The recombinant protein was similarly treated and the protein concentration determined by extrapolation from the standard curve as indicated in (Appendix B; Figure B.1). The readings were prepared in triplicate and the average obtained.

A. 11 Isolation of human peripheral blood granulocytes (PMNs)

Human peripheral blood granulocytes (PMNs) where isolated from peripheral blood, collected by venipuncture from healthy adult volunteers. Peripheral blood was collected by venipuncture from healthy adult volunteers. Informed consent was obtained from the volunteers. The anticoagulated blood (lithium heparin) was layered onto a histopaque gradient consisting of 2 layers: lymphocyte separation medium (PAA Laboratories, Pasching, Austria) on the top and histopaque 1119 (Sigma-Aldrich, Deisenhofen, Germany) at the bottom prior to centrifugation at 800g for 20 minutes. The granulocyte-rich layer of histopaque 1119 was collected and washed once in PBS (PAA Laboratories). The PMN pellet was resuspended in RPMI 1640 medium (Sigma-Aldrich, Deisenhofen, Germany) and further fractionated on a discontinuous percoll (Amersham Biosciences, Freiburg, Germany) gradient consisting of layers with densities of 1105 g/mL (85%), 1100 g/mL (80%), 1093 g/mL (75%), 1087 g/mL (70%), 1081 g/mL (65%) percoll. After centrifugation for 20 minutes at 800g, the interface between the 80% and 85% percoll layers was collected and washed twice in PBS. PMN purity was assessed by size and granularity on flow cytometry (BD Bioscience). The isolated Neutrophils were cultured at 37°C (5% CO_2) incubator, using complete medium (RPMI 1640 medium supplemented, 10 mM HEPES, 10% heat inactivated fetal bovine serum (all from Sigma-Aldrich, Steinheim, Germany),

4 mM Lglutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all purchased from Biochrom, Berlin, Germany).

B. 1 Bradford standard curve

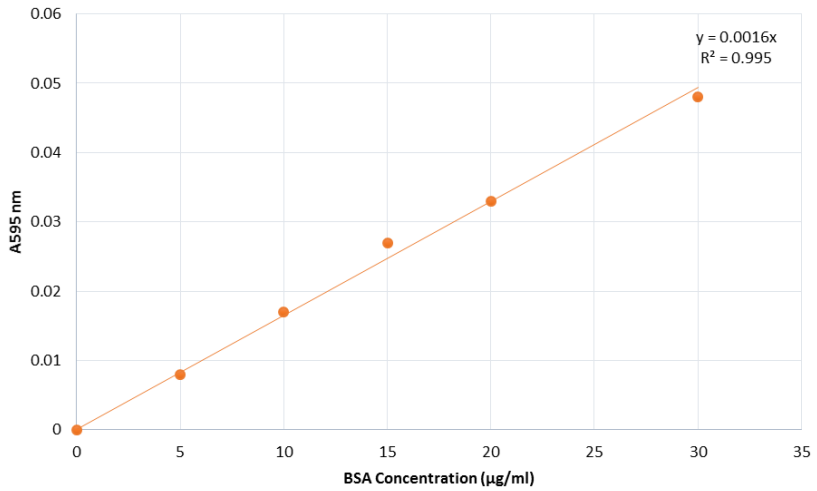


Figure B. 1 Bradford standard curve for protein concentration determination
Bovine serum albumen (BSA) standards of concentration ranging from 0 to 30 µg/ml were prepared and absorbance was read at 595 nm using (Biorad, U.S.A). The linear equation: y = 0.0016x; R^2 = 0.995 was used to calculate the protein concentration.

B. 2 Phosphate standard curve

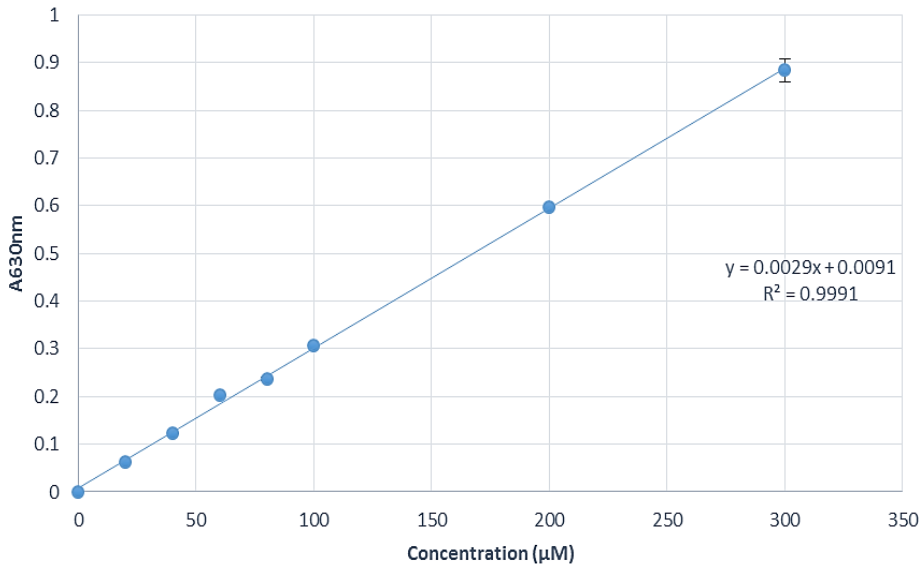
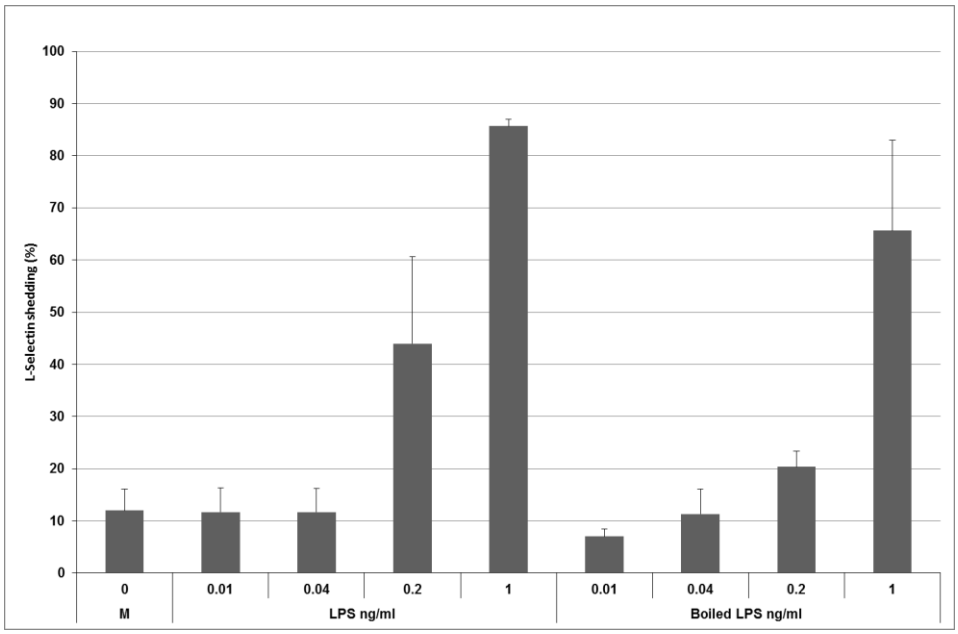


Figure B. 2 Phosphate standard curve for ATP hydrolysis analysis
Phosphate standards of concentration ranging from 0 to 300 µM were prepared and absorbance was read at 630nm using (Biorad, U.S.A). The linear equation: y = 0.0029x + 0.0091; R^2 = 0.9991 was used to calculate the protein concentration.

B. 3 Evaluating the effect of boiling on LPS

A)



B)

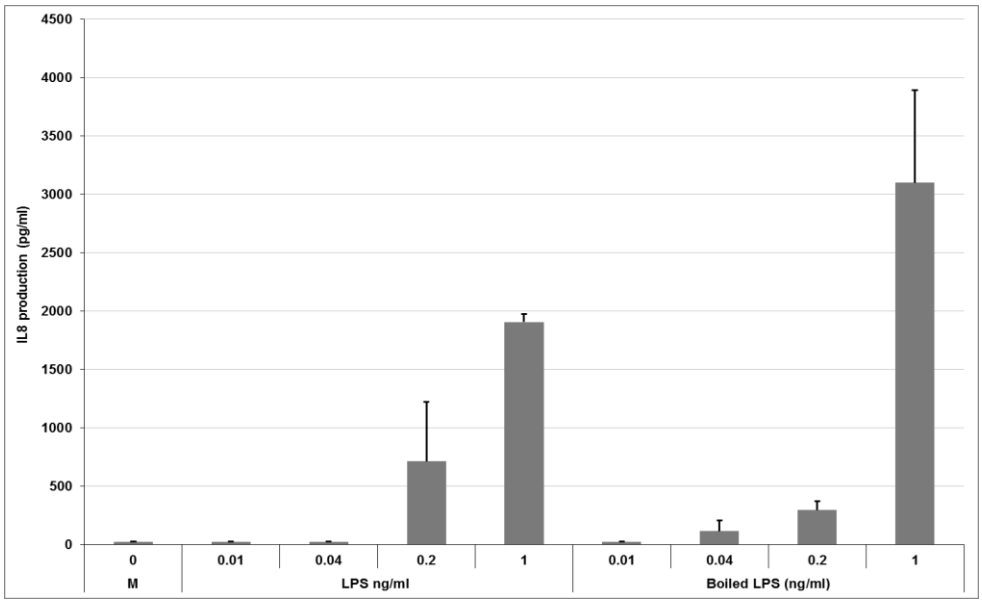
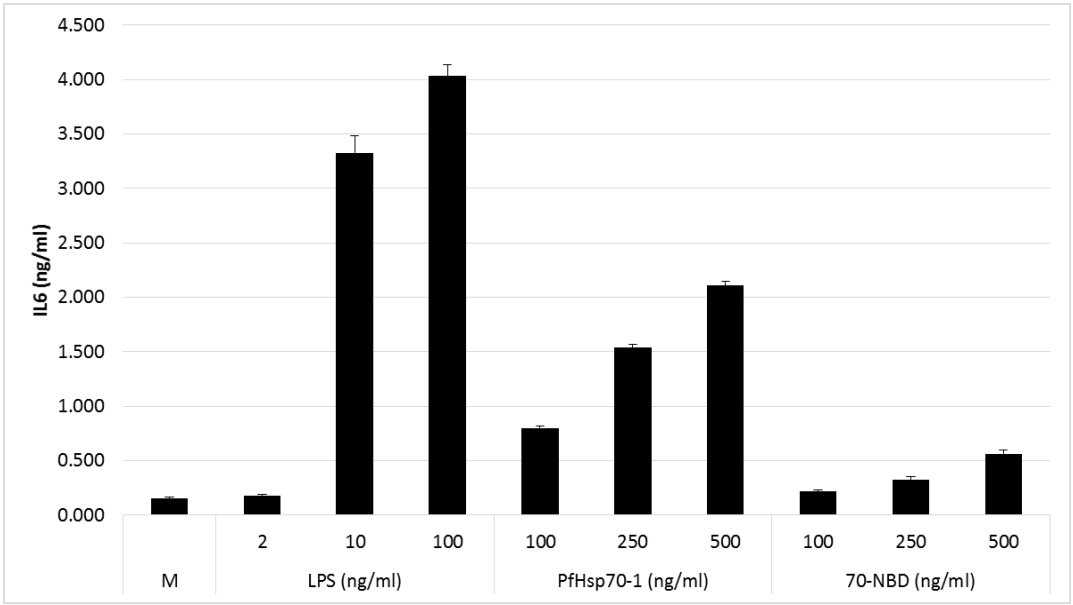


Figure B.3 LPS stimulatory activity is not hinderd by boiling
Equal amounts of boiled and non boiled LPS was used to stimulate equivalent amounts of PMN cells. Boiling LPS does not affect IL8 release and L-selectin shedding by PMN. L-selectin shedding analysis on PMN stimulated with boiled and non boiled LPS (A). IL8 expression by PMN challenged with boiled and non boiled LPS (B).

B. 4 Recombinant proteins produced in *E. coli* XL1Blue cells induce IL6 and IL12 production on DCs

A)



B)

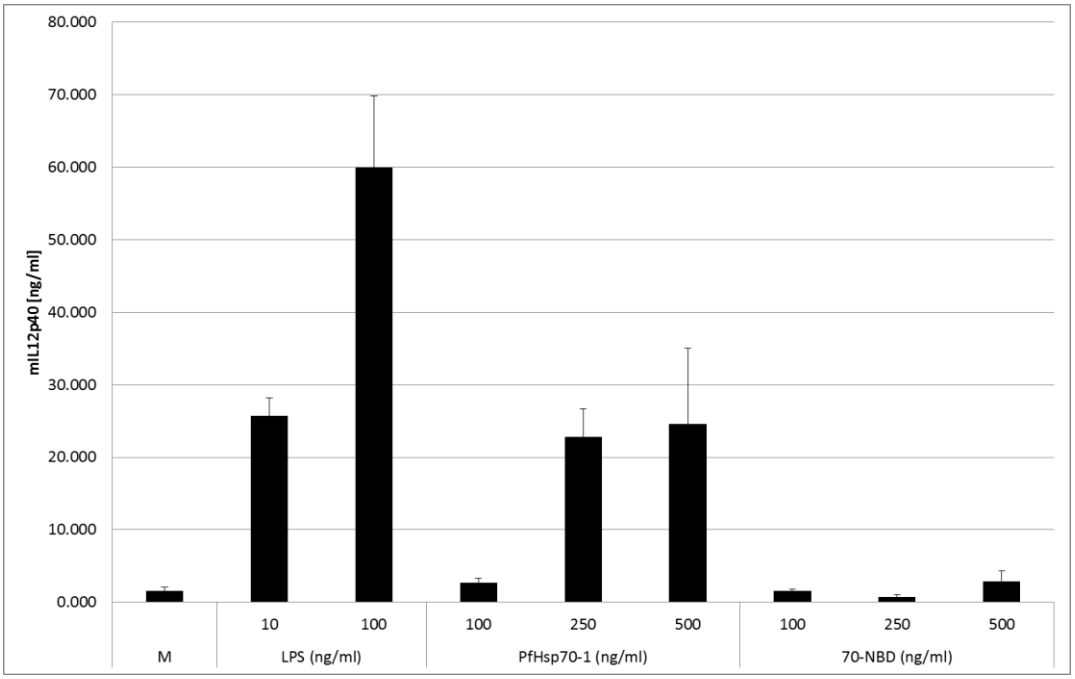
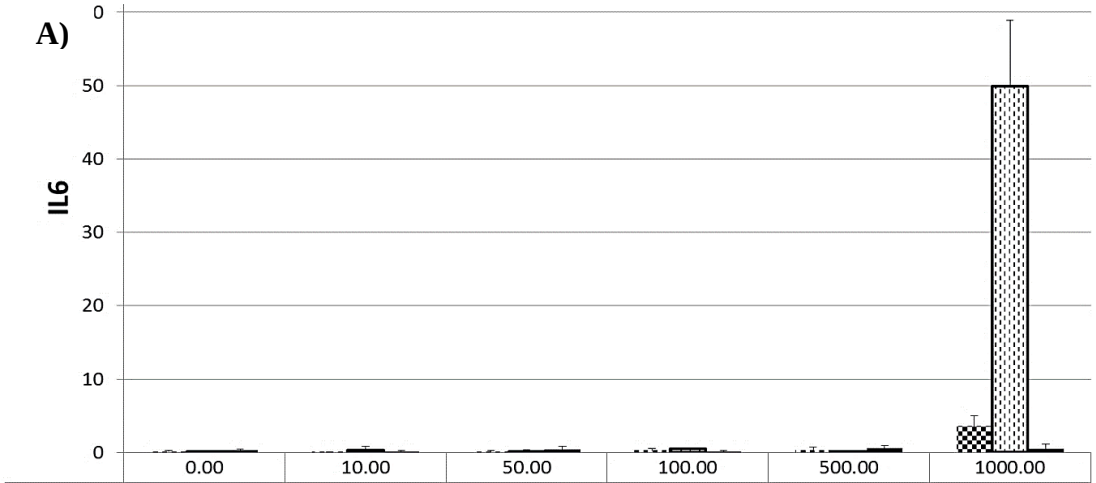
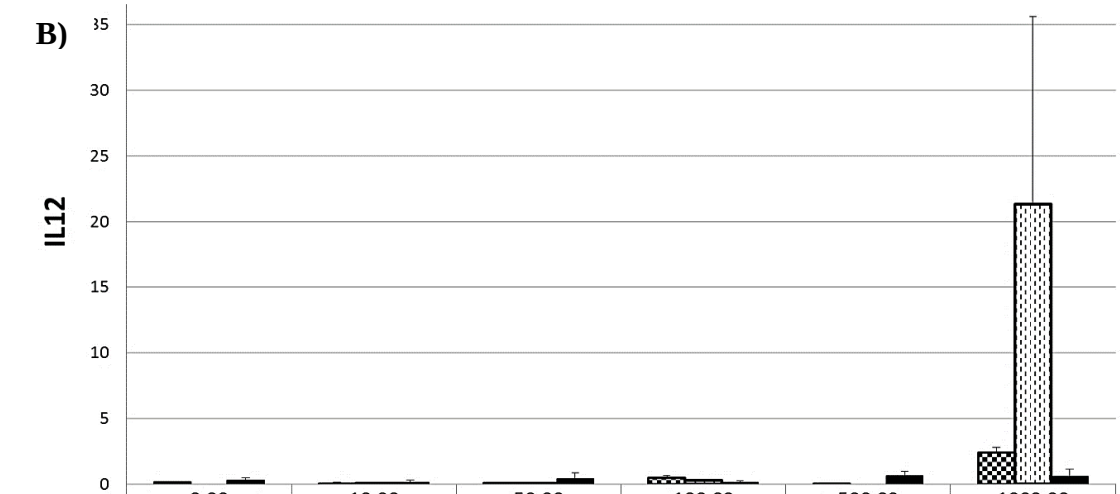


Figure B.4 Protein purified from *E. coli* XL1 Blue stimulates IL6 and IL12 production
Equal amounts of PfHsp70-1 and PfHsp70-1_{NBD} (70-NBD) produced in *E. coli* XL1 Blue were used to stimulate equivalent amounts of DCs. PfHsp70-1 produced *in E. coli* XL1 Blue stimulated IL 6 (A) and IL12 (B) production. LPS were used as positive control. M, unstimulated cells.

B. 5 Recombinant proteins produced in *E. coli* ClearColi BL21 cells do not induce cytokine expression when on DCs



	0.00	10.00	50.00	100.00	500.00	1000.00
PfHsp70-1	0.152333333	0.043	0.111666667	0.29	0.339666667	3.583333333
70-NBD	0.043	0.315	0.146	0.544	0.048	49.907
PfLDH	0.304	0.109	0.373	0.106	0.599	0.544



	0.00	10.00	50.00	100.00	500.00	1000.00
PfHsp70-1	0.153333333	0.056666667	0.078	0.473	0.002	2.429
70-1 NBD	0.000	0.006	0.050	0.306	0.000	21.325
PfLDH	0.304	0.109	0.373	0.106	0.599	0.544

Figure B.5 Cytokine production on murine DCs cells incubated with protein produced in *E. coli*
Equal amounts of murine macrophages cells were co-cultured with PfHsp70-1, PfHsp70-1_{NBD} (70-NBD) and PfLDH produced in *E. coli* ClearColi BL21 with the indicated amounts (0, 10, 50, 500 and 1000 ng/ml). IL6 (A) and IL12 (B) were analysed following the manufactures standards. LPS were used as positive controls for TLR 2 and TLR4 cells respectively.

B. 6 Cytokine induction analysis on TLR2 and TLR4 HEK293 cells treated with PfHsp70-1 produced in *E. coli* ClearColi BL21 and *Brevibacillus choshinensis*

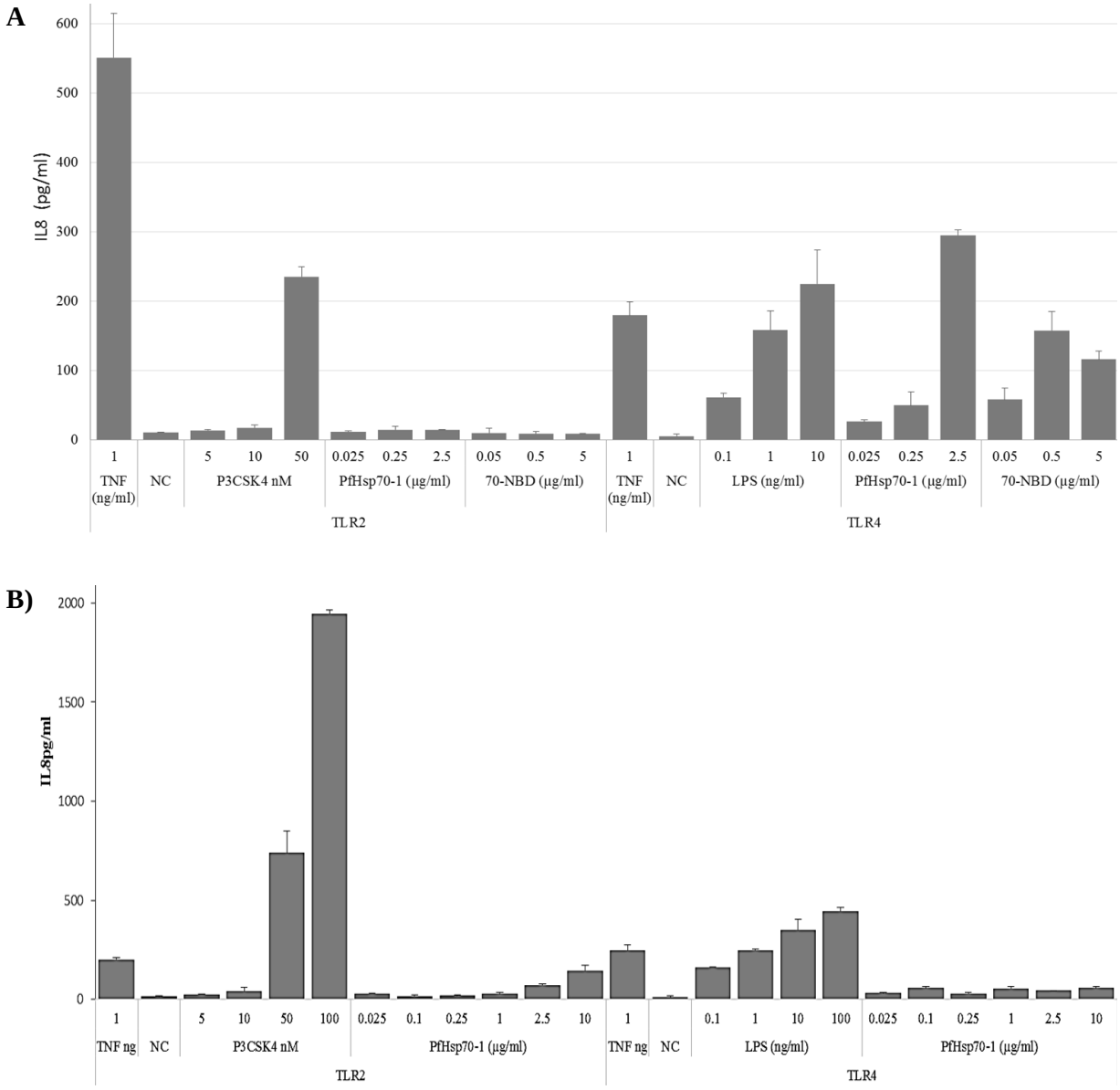


Figure B.6 Analysis of IL8 production on TLR2 and TLR4 HEK293 cells treated with Pf1Hsp70-1 produced in *E. coli and B. choshinensis*

Equal amounts of PfHsp70-1 and PfHsp70-1_{NBD} (70-NBD) produced in *E. coli* were used to stimulate equivalent amounts of TLR2 and TLR4 transfected HEK293 cells (A). PfHsp70-1 produced *in Brevibacillus choshinensis* was used to stimulate equivalent amounts of TLR2 and TLR4 cells (B). P3CSK4 and LPS were used as positive controls for TLR 2 and TLR4 cells respectively. NC, unstimulated cells. The cells were also stimulated with TNF.

B. 7 PfHsp70-1_{NBD} produced in *E. coli* XL1Blue cells

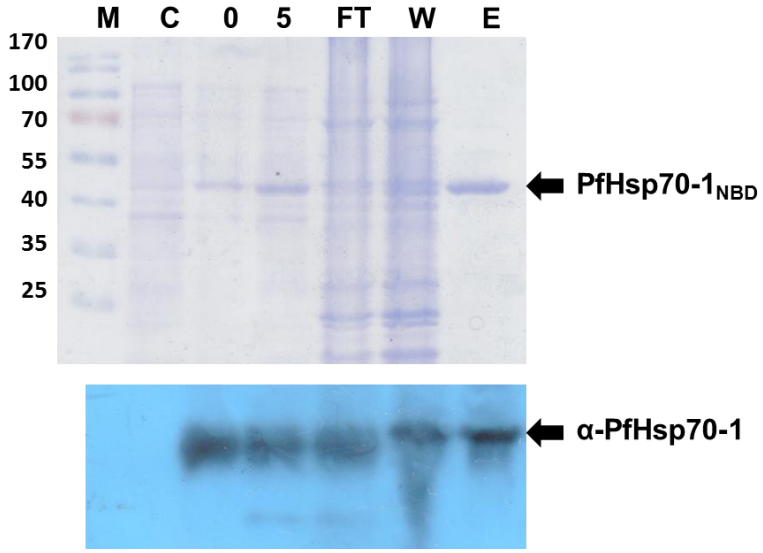


Figure B.7 Successful expression and purification of PfHsp70-1_{NBD} in *E. coli* XL1Blue cells
PfHsp70-1_{NBD} (45 kDa) was over-expressed in *E. coli* XL1 Blue cells. The proteins were successfully purified by nickel-affinity chromatography. PfHsp70-1_{NBD} expression and purification were analysed by 12.5% SDS-PAGE (upper panel). Lane C, untransformed cells; lane 0, pre-induction; and lane 5, 5 hours post-IPTG induction. M, molecular weight marker of recombinant proteins. Lane FT and W, are flowthough non-bound protein and wash buffer samples, respectivelty. Lane E, the eluted protein samples Western blot analysis α-PfHsp70-1 polyclonal antibody (1: 2000) was used to confirm the presence of the PfHsp70-1_{NBD} (lower panel).

Appendix C: Specialised reagents

Table C. 1 List of materials and specialised reagents

Reagent	Supplier
Acetic acid	Merck, Germany
Adenosine triphosphate	Sigma, U.S.A
Agarose	Whitehead scientific, South Africa
Ammonium molybdate	Merck, Germany
Ammonium persulphate	Merck, Germany
Ampicillin	Sigma, U.S.A
Bovine serum albumin	Sigma, U.S.A
Bromophenol blue	Sigma, U.S.A
Calcium chloride	Merck, Germany
Chloramphenicol	Sigma, U.S.A
CL-Xposure™ Film	Pierce, USA
Coomasie brilliant blue R250	Merck, Germany
Diethiothreitol	Sigma, U.S.A
DreamTaq master mix	ThermoScientific, U.S.A
Ethidium bromide	Sigma, U.S.A
Glacial acetic acid	Merck, Germany
Glycerol	Merck, Germany
Glycine	Merck, Germany
Imidazole	Sigma, U.S.A
Isopropyl-1-thio-D-galacopyranoside	Sigma, U.S.A
Lysozyme	Merck, Germany
Magnesium chloride	Merck, Germany
Methanol	Merck, Germany
Monoclonal anti-His6-Peroxidase antibodies	Sigma, U.S.A
Ni-chelating sepharose	Amersham, U.S.A
Nitrocellulose membrane	Pierce, U.S.A

Specialised reagents

PagerRuler Prestained Protein Ladder	ThermoScientific, U.S.A
Peptone	Merck, Germany
Reagent	**Supplier**
Phenylmethylsufonyl fluoride	Sigma, U.S.A
Polyacrylamide	Merck, Germany
Polyethylene glycol 2000	Sigma, U.S.A
Polyethylenimine	Sigma, U.S.A
Ponceau S	Sigma, U.S.A
Potassium chloride	Merck, Germany
Potassium dihydrogen phosphate	Merck, Germany
Proteinase-K	Sigma, U.S.A
QuikChange II site-directed mutagenesis kit	Stratagene, U.S.A
Rapid ligation buffer	Promega, Germany
Restriction enzymes	Fermentas, U.S.A
Snakeskin™ pleated dialysis tubing	Pierce, U.S.A
Sodium chloride	Merck, Germany
Sodium dodecyl sulphate	Merck, Germany
Sodium hydroxide	Merck, Germany
TEMED	Sigma, U.S.A
Tris	Merck, Germany
Tryptone	Merck, Germany
Tween 20	Merck, Germany
Yeast extract powder	Merck, Germany
β-mercaptoethanol	Sigma, U.S.A