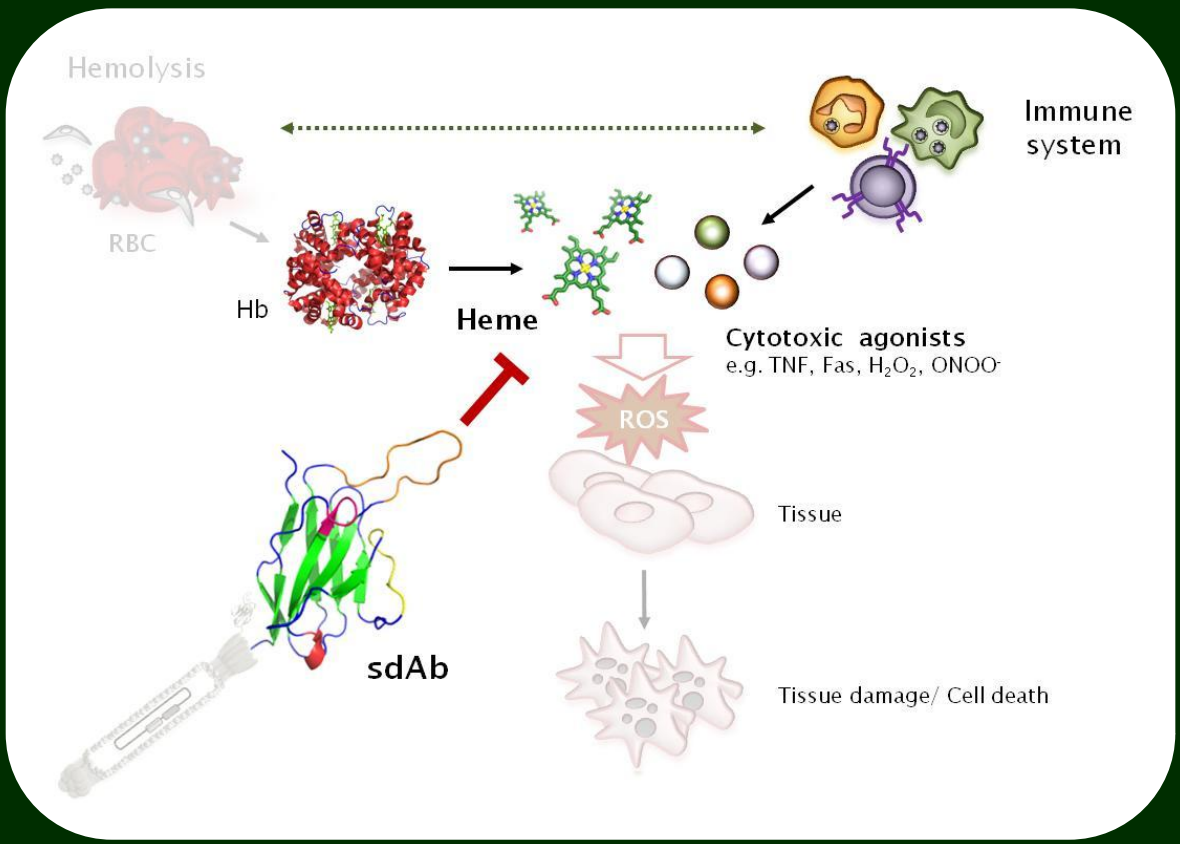
Targeting Heme with Single Domain Antibodies

Zélia Licínia Ferreira Gouveia



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
June, 2015

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER /UNL

Knowledge Creation

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Research work coordinated by:

INSTITUTO GULBENKIAN DE CIÊNCIA

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Targeting Heme with Single Domain Antibodies

Zélia Licínia Ferreira Gouveia

Supervisor:

Dr. Miguel P.Soares
Instituto Gulbenkian da Ciência

Co-Supervisor:

Dr. Smilja Todorovic
Instituto de Tecnologia Química e Biológica

PhD fellowship financed by Fundação para a Ciência e Tecnologia (Apoio financeiro da FCT e do FSE no âmbito do Quadro Comunitário de apoio, BD: SFRH/BD/44828/2008)

Oeiras, Junho, 2015

Acknowledgements

Firstly and primarily I would like to express my most sincere gratitude to my Ph.D supervisor Dr. Miguel P. Soares for accepting me as his Ph.D student and to trust me this Ph.D project. I want to thank him for his help, advices and for non-stop teaching and extremely helpful and constructive scientific discussions. Today, I am the scientist that you help to build, thank you very much! I extremely grateful to Dr. Miguel P. Soares for his understanding and support, in a particular period of my life in which I had to take several weeks of my work time to be and support my family as we were having a extremely difficult time in our life. For all of this and more, thank you very much!

I am grateful to Dr. Smilja Todorovic, for giving me the opportunity to join in her laboratory and for accepting me as Ph.D student, for her advices, support and precious and fundamental help in Resonance Raman spectroscopy.

I also want to thank to all the present and past members of Inflammation groups for their help, support, discussion and the most diverse moments spent together. A special thanks to Sofia, for her advices, support and many other things. To Viktória Jeney, for all the help and teach in the begining of this project and for the beautiful words and advices. Thanks the wonderful person, Ramus, for showing me that the comprehension and the calm can be your best friends in the moments that everything seems to be under tumult. Thanks to Raffi, for all your help. Sílvia, thank you very much for been always so friendly. To Rita Carlos, Susana Ramos and Sebastian Weiss thanks for the comments and the correction of the introduction part of the thesis.

To my previous group member and now good friend Ana Cunha, my thanks for being always present and supportive and for all her scientific and non scientific help.

To my good friend Ana Rita Mateus, my thanks for the support and for always been presence in the good or less good moments. Ana Regalado, my sincere and most adorable friend, thank you very much for being always present and willing to listen and share ideas and advices.

I am also grateful to the people involved in the many collaborations that support this work (chapter 2), including
Dr. João Gonçalves from Faculdade de Farmácia e Instituto de Medicina Molecular, for receiving me in his research group for almost two years and to supervise my work in the development of single domain antibodies against heme using phage display technology. Thanks also to all his group members, for their help, support and time.
Dr. Frederico Aires-da-Silva, from Technophage for providing me many useful suggestions about Phage display technology and many other things. Also, for all the scientific discussions and for being always available to take time to teach me and sharing his scientific knowledge.
Dr. Claúdio Gomes from Instituto tecnologia Química e Biológica, for his kindness, availability and all his scientific help to performed spectroscopic techniques
Dr. Olga Iranzo from Instituto tecnologia Química e Biológica, for her precious help in the biotinylation of Heme.
Dr. Iqbal Hamza from the University of Maryland, for his kindness, availability and discussion on this work. Also, for receiving me his laboratory and providing me all the material and support required to perform HPR assay. To all his group members and in special Tamika Samuel and Carine White for their kindness, availability and scientific or non scientific help, as well as Jason Sinclair and Xiaojing Yuan for their kindness and availability.
And to all the other collaborators, my thanks.

I am also grateful to my Thesis committee, Dr. Alekos Athanasiadis, IGC and Dr. Miguel Teixeira, ITQB.

I want to thank to Instituto Gulbenkian da Ciência and all IGC community, for providing an amazing scientific environment.

All this work was only possible because I have amazing people supporting me at all the moments, my loved companion and my family and of course my good friends.

I want to thank my beloved family and specially my mother, Felismina Pereira Ferreira and my father, José da Assunção Gouveia, for so many things that there is no space in this thesis to mention them. My most and important thank you. Ao meu querido pai, José da Assunção Gouveia, o meu Muito Obrigada para todo o sempre! Se hoje defendo esta tese é sem dúvida graça a ti e à mãe!

To my companion Pierre Crozet, first I would like to thank you for being such an amazing friend and for all the love demonstrated and shared. Thank you for all the support, help and comments/discussions in non and scientific fields. Thanks for helping me in my thesis, correcting/commenting it. I am also grateful to his amazing and adorable family to receive me like family. Merci à tous, et surtout à Joëlle Crozet-Vaugelade, Jean Jeacques Crozet et Elisabeth Crozet.

I would like to acknowledge the Fundação para a Ciência e Tecnologia (Apoio financeiro da FCT e do FSE no âmbito do Quadro Comunitário de apoio, BD: SFRH/BD/44828/2008), Instituto Gulbenkia da Ciência and Inflammation Group of Dr. Miguel P. Soares, who have finantially supported my Ph.D Work.

I would like to apologize in advance to the many people that I might not have mention in this section but for sure help me along these years to develop this work. For all of them, my sincere thanks.

I would like to finalize with these two popular proverbs “Não importa o tamanho da montanha, ela não pode tapar o sol (Doesn’t matter the size of the mountain, she cannot be cover by the sun)” and “Quando as nuvens passam o Sol brilha” (When

the clouds pass, the sun shines) and by dedicating this thesis to my family, but specially to my beloved father José da Assunção Gouveia, who will always be with us and to my beloved mother Felismina Pereira Ferreira, without them this thesis could not be possible.

My eternal thanks!

Z.Gouveia

Abreviations

[wav(x), wav(y); Eg]; Waving
5c; Five coordinate
6c; Six coordinate
aa; amino acids
Ab(s); Antibodie (s)
ABCB10 or ABC-me; ATP-binding cassette sub-family B member 10
ABCB6; ATP-binding cassette sub-family B member 6
ABCG2; ATP-binding cassette transporter G2
ACN; Acetonitrile
ACTH; Adrenocorticotropic hormone
Ala, A; Alanine
ALA; δ-aminolevulinic acid
ALAS; δ -aminolevulinate synthase
ALAS-E; δ -aminolevulinate synthase erythroid-specific (=ALAS2)
ALAS-N; Delta-aminolevulinate synthase nonspecific (=ALAS 1)
ANOVA; Analysis of variance
ApoE; Apolipoprotein E
Arg, R; Arginine
Asp, N; Asparagine
ATP; Adenoside triphosphate
ATPase; Adenylpyrophosphatase
ATR-FTIR; Attenuated total reflection Fourier transform infrared
BACH1; Basic leucine zipper transcription factor 1
Bach2; Basic leucine zipper transcription factor 2
BCP; Breast cancer resistance protein
Biotin; N-[-5- (Hydrazinocarboxy) pentyl]-D-biotinamide
Blimp-1; B lymphocyte-induced maturation protein-1
BR; Bilirubin
BV; Biliverdin
BVR; Biliverdin reductase
BW; Body weight
CCD; Charge coupled device
ccm; System I cytochrome c maturation
CcmE; Cytochrome c maturation E
CD; Circular dichroism
CD14; Cluster of differentiation 14
CD163; Cluster of differentiation 163
cDNA; Complementary DNA
CDR; Complementarity-determining regions
cGMP; Cyclic guanosine monophosphate
CH; Heavy chain constant domain
ChEBI; Chemical entities of biological interest
Cl; Chloride
CL; Liht chain constant domain
CLP; Cecal ligation and puncture
CN; Cysteine- asparagine
CO; Carbon monoxide
CO_2; Carbon dioxide
CoPP; Cobalt protoporphyrin IX
COPRO III; Coproporphyrinogen III

CORMs; Monoxide releasing molecules
CPOX; Coproporphyrinogen oxidase
CS; Cysteine-serine
Ctrl; Control sdAb
CY; Cyanine dyes
Cys, C; Cysteine
Cytc; Cytochrome c
DAPI; 4',6-diamidino-2-phenylindole
DETAPAC; Diethylenetriaminepentaacetic acid
DeutP; Iron deuteroporphyrin IX
DHB; 2,5-dihydroxybenzoic acid
DMEM; Dulbecco's modified eagle medium
DMF; Dimethylformamide
DMSO; Dimethylsulfoxide
DNA; Deoxyribonucleic acid
dom, A2u; Doming
***E.coli*;** *Escherichia cole*
EDTA; Ethylenediaminetetraacetic acid
EEA; Early endosomes
ELISA; Enzyme-linked immunosorbent assay
ER; Endoplasmatic reticulum
ERK-2; Extracellular signal-regulated kinases 2
Fab(s); Antigen binding fragment(s)
FABP; Fatty acid binding protein
FBS; Fetal bovine serum
Fc; Constant fragment region
FcgRs; Fc receptors
FcRn; Neonatal Fc receptor
Fe; Iron
$Fe_2(SO_4)_3$; Iron (III) sulfate
Fe^{2+}; Ferrous ion
Fe^{3+}; Ferric ion
FECH; Ferrochelatase
FePP; Hemin
FePPCH3; Iron protoporphyrin IX dimethyl ester chloride
FF; Fast flow
FITC; Fluorescein isothiocyanate
FLVCR2; Feline leukemia virus subgroup C receptor 2
FR; Framework region
Ft; Ferritin
FtH; Heavy/heart chain
FtL; Light/liver chain h
Fv; Variable fragment domain
Gly, G; Glycine
GaPP; Gallium protoporphyrin IX
GATA1; GATA binding protein 1 (globin transcription factor 1)
GSH; Reduced glutathione
GSSG; Oxidized glutathione
GST; Glutathione S-transferase B
H_2O_2; Hydrogen peroxide
H_2SO_4; Sulfuric acid
HA; Human influenza hemagglutinin
HABA; 4'-hydroxyazobenzene-2-carboxylic acid
HATU; 2- (1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate methanaminium
Hb; Hemoglobin
HBC; Heme buffering capacity
HbC; Hemoglobin C
HbE; Hemoglobin E

HBM; Heme-binding motifs
HBP23; Heme-binding protein 23 kDa
HbS; Hemoglobin S
HCl; Hydrochloric acid
HCP-1; Heme carrier protein
HD; Heme depleted serum medium
HDL; High density lipoprotein
HEK293; Human embryonic kidney 293
Heme; Protoheme IX
Hep2; Human epithelial type 2
HEPES; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1; Hypoxia inducible factor-1
His, H; Histidine,
HMB; Hydroxymethylbilane
HMBS; Hydroxylmethylbilane synthase
HO-1; Heme oxygenase 1
HOCl; Hypochlorous acid
Hp; Haptoglobin
HPLC; High performance liquid chromatography
HPX; Hemopexin
HRG-1; Heme-responsive gene-1
HRM; Heme regulatory motifs
HRP; Horseradish peroxidase
HSA; Human serum Albumin
HSAH; Human serum albumin bound to heme
HSP90; Heat-shock protein 90
ICAM; Intercellular adhesion molecule
IFNβ; Interferon beta
Ig; Immunoglobulin
IL; Interleukine
Ile, I; Isoleucine
iNOS; Inducible nitric oxide synthase
IPTG; Isopropyl β-D-1-thiogalactopyranoside
IκB; Inhibitor of κB
JNK; c-Jun N-terminal Kinase
K^+; Potassium ion
KC; keratinocyte derived chemokine
KD; Dissociation constant
LB; Luria Broth
LDL; Low density lipoprotein
Leu, L; Leucine
LIPR; Leucine-isoleucine-proline-arginine
LRP; Low-density lipoprotein receptor-related protein/CD91
LS; Low spin
LTB4; Leukotriene B4
Lys, K; Lysine
m/z; Mass-to-charge ratio
mAb; Monoclonal Ab
MALDI; Matrix-assisted laser desorption/ionization
MAPKs; Mitogen-activated protein kinases
Mb; Myoglobin
MEL; Murine erythroleukemia
Met, M; Methionine
MetHb; Methemoglobin
MFRN1; Mitoferritin 1
$MgCl_2$; Magnesium chloride
Mn^{2+}; Manganese ion
Mø(s); Macrophage(s)
MPO; Myeloperoxidase
mRNA; Messenger ribonucleic acid
MS; Mass spectrometry
mtDNA; Mitochondrial DNA

MyD88; Myeloid differentiation primary response gene 88
NAC; N-acetylcysteine
NaCl; Sodium chloride
NADPH; Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$; Sodium hydrogen carbonate
NaOH; Sodium hydroxide
NAR; (IgNAR) variable domain
NETs; Neutrophil extracellular traps
NF-κB; Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3; Leucine rich repeat containing family pyrin domain containing 3 inflammasome
NO; Nitric oxide
NOD; Nucleotide-binding oligomerization domain receptor
NOX; NADPH Oxidase
NPAS2; Neuronal PAS domain-containing protein 2
NRF2; Nuclear factor (erythroid-derived 2)-like 2
O_2; Dioxygen
O^{2-}; Superoxide ion
ON; Overnight
p22HBP; Heme-binding protein 22
PBG; Porphobilinogen
PBS; Phosphate buffered saline
PCD; Programmed cell death
PCR; Polymerase in chain reaction
PEG; Polyethylene Glycol
PG; Prostaglandins
PGBS; Porphobilinogen synthase
Phe, F; Phenylalanine
PHZ; Phenylhydrazine
PI3K; Phosphoinositide 3-kinase
PKB or Akt; Protein kinase B
PKC; Protein kinase C
pNPP; para-Nitrophenylphosphate
PP IX; Metal free protoporphyrin IX
PP; protoporphyrin
PPG IX; Protoporphyrinogen IX
PPIX; Protoporphyrin IX
PPOX; Protoporphyrin oxidase
Pro, P; Proline
pro,A1u; Propellering
PRR; Pattern recognition receptor
Prx I; Peroxiredoxin
RBC; Red blood cells
RIP; Receptor-interacting protein
RIPK1; Receptor-interacting serine/threonine kinase 1
ROS; Reactive oxygen species
RR; Resonance Raman
RT; Room temperature
RU; Resonance units
ruf, B1u; Ruffling
Ser, S; Serine
S/N; Sinal to noise ratio
SA; Succinylacetone
sad, B2u; Saddling
SCD; Sickle cell disease
scFv; Single chain Fv
SD; Standard deviation
sdAb(s); Single domain Ab(s)
SDS-PAGE; Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM; Standard error of the mean

Ser; S; Serine

sGC; Guanylyl cyclase

SIRS; Systemic inflammatory response syndrome

SnPP; Tin protoporphyrin IX

SOD; Superoxide dismutase

SOUL; Heme-binding protein 2

SPR; Surface plasmon resonance

SyK; Spleen tyrosine kinase

TBS; Tris-buffered saline

TGF-β; Transforming growth factor β

Thr, T; Threonine

TLR; Toll like receptor

TMB; 3,3',5,'-Tetramethylbenzidine

TNF; Tumor necrosis factor

TOFMS; Time-of-flight mass spectrometry

Trp, W; Tryptophan

TRIF; TIR (Toll–IL-1 receptor)-domain-containing adapter-inducing interferon

Tyr, Y; Tyrosine

t-α1m; Truncated form of α -microglobulin

URO III; Uroporphyrinogen III

UROD; Uroporphyrinogen III decarboxylase

UROS; Uroporphyrinogen III co-synthase or isomerase

Val, V; Valine

VH; Heavy chain variable domain

VHH; heavy-chain-only antibody (Ab) V(H)

VL; Light chain variable domain

V-NAR; Variable NAR domain from shark

VWF; Willebrand factor

WBP; Weibel-Palade bodies

XIAP; X-chromosome-linked inhibitor of apoptosis

ZnPP; Zinc protoporphyrin IX

α1m; α -microglobin

β-ME; β-mercaptoethanol

Preface

This thesis “Targeting heme using single domain antibodies” aimed to develop methodologies that would overcome the current limitations in the detection and quantification of “free heme”, *i.e*. redox active heme that is loosely bound to proteins or to other biological molecules. Here, we described the generation of single domain antibodies specific against heme using phage display technology and characterized them by different techniques such as affinity determination, antigen binding specificity, structural analysis (spectroscopy techniques), *in vitro* and *ex-vivo* actions. These sdAbs were further used to develop methodologies for the quantitative and qualitative analysis of “free heme” and its cellular detection as well as to modulate heme reactivity.

This research work was performed mainly in Instituto Gulbenkian de Ciência under the scientific supervision of Dr. Miguel P. Soares. While, the development of the sdAbs was performed in collaboration and under the supervision of Dr. João Gonçalves in Faculdade de Farmácia da Universidade de Lisboa and Dr. Frederico Aires-da-Silva in Technophage from Instituto de Medicina Molecular da Universidade de Lisboa. Spectroscopy work was performed in the Instituto tecnologia Química e Biológica. These include Resonance Raman spectroscopy under the scientific supervision of the co-supervisor Dr. Smilja Todorovic and CD and FTIR spectroscopy in collaboration and under the scientific supervision of Dr. Claúdio Soares. The sdAb affinity measurements were also performed in Instituto tecnologia Química e Biológica with the supervision of Dr. Frederico Aires-da-Silva. The flow cytometry and immunofluorescence assay was performed by Ana Rita Carlos, IGC and ascorbate oxidation designed Roland Stocker, University of New South Wales, Australia.

The thesis is organized in three chapters, preceded by a Portuguese abstract translated in English. In the chapter 1, general introductory review on the subject is provided. In the chapter 2 the general and most significant results obtained during the research period are shown and discussed. In the chapter 3 a general discussion

integrating all work and future perspectives are presented. In the appendix is presented and discussed supplementary information about a preliminary work to generate constructs, namely dimers of sdAbs and sdAbs containing an additional region of heme recognition (CDR), based on the sdAbs developed in chapter 2, namely sdAbs 1A6 and 2H7.

During my PhD project was able to participate in the elaboration of a review manuscript entitled “Heme Cytotoxicity And The Pathogenesis of Immune Mediated Inflammatory Diseases.” (DOI:10.3389/fphar.2012.00077) from Rasmus Larsen, Zélia Gouveia, Miguel P. Soares and Raffaella Gozzelino, published in the Journal Frontiers in pharmacology; 2012, with a relevant topic for the present work of this thesis.

Sumário:

Hemo, *i.e.* ferro (Fe) contido na porfirina IX, funciona como grupo prostético numa variedade de hemoproteins que exercem funções biológicas vitais e portanto, essenciais para sustentar a vida. Hemo é uma molecula extremamente reactiva, podendo participar em reacções redox e presumivelmente por esse motivo deve ser sequestrado no interior de um bolso hémico nas hemoproteínas, para controlo da sua reactividade. Contudo, sob condições de stress biológico, as hemoproteínas podem libertar o seu grupo prostético formando "hemo livre" que liga-se fracamente às proteínas, ou outras moléculas adquirindo presumivelmente actividade redox descontrolada. Para além disso, um crescente número de evidências suporta a noção de que o "hemo livre" pode comportar-se como um agente vasoativo, pro - inflamatório e citotóxico quando libertado de um subconjunto de hemoproteínas como por exemplo, a extracelular hemoglobina gerada durante condições hemolíticas. Assim sendo, eventualmente o "hemo livre" contribui para a patogénese de doenças inflamatórias imuno-mediadas associadas com hemólise. As actuais limitações das abordagens metodológicas que possibilitem a análise quantitativa e qualitativa do "hemo livre" impedem a determinação inequívoca dos seus efeitos patológicos, tal como o seu eventual tratamento terapêutico. Para superar estas limitações, usamos a technologia de exibição de fagos para gerar um painel de anticorpos de domínio único ('single domain antibodies', sdAbs), nomeadamente cadeia variável leve (VL), que reconhecem específicamente "hemo livre" após libertação das respectivas hemoproteínas.

O sdAb 2H10 foi selecionado, uma vez que é o mais específico para o "hemo livre" e a sua ligação ao hemo foi caracterizada usando as várias técnicas espectroscopicas (absorção no UV-visível, Raman ressonante, Dicroísmo circular). Estas técnicas demonstraram a presença de um hemo férrico seis coordenado de baixo 'spin', com uma cisteína como quinto ligando e, possívelmente uma lisina ou tirosina como o sexto ligado. Para além disso, a estructura secondária do sdAb, composta principalmente por folhas β antiparalelas, sofre alterações

conformacionais para acomodar o hemo. Foi também demonstrado que estes sdAbs podem ser usados para detectar “hemo livre” em células humanas através de técnicas de imunofluorescência ou citometria de fluxo, tal como no plasma utilizando um ensaio ‘enzyme linked immunoabsorbant assay’ (ELISA), desenvolvido a partir dos sdAbs.

Para verificar a existência do “hemo livre” no plasma, utilizámos um modelo experimental previamente estabelecido usando a fenilhidrazina (PHZ) para induzir hemólise aguda em murganhos. Neste ensaio o “hemo livre” não é detectado no plasma, mesmo quando os níveis de hemo total e de absorção celular do hemo aumentam, enquanto que a capacidade do plasma para ligar o hemo diminui transitoriamente. Eventualmente, a elevada capacidade de tampão do plasma é assegurada por proteínas, ou outras moléculas biológicas, proporcionando um mecanismo preciso e regulador para a manutenção da homeostase sistémica do hemo. Demonstrámos ainda que a ligação de sdAb 2H10 ao hemo reduz a sua actividade redox, ainda mais eficientemente do que o seu eliminador plasmático, albumina, uma acção que possivelmente possibilitará a inhibição dos efeitos patológicos do hemo.

Em resumo, nós demonstramos que estes sdAbs podem ser usados para ultrapassar as actuais limitações relativas à análise quantitativa e qualitativa do “heme livre” em condições hemolíticas, ou noutras condições biológicas associadas presumivelmente com a libertação do hemo das hemoproteinas. Propõe-se a utilização dos sdAbs para fins terapêuticos, nomeadamente do sdAb 2H10, na captação do “hemo livre”, tal como na modulação da sua actividade redox em patologias associadas com hemólise.

Abstract:

Heme, i.e. iron (Fe) protoporphyrin IX, functions as a prosthetic group in a variety of hemoproteins that participate in vital biologic functions essential to sustain life. Heme is a highly reactive molecule, participating in redox reactions, and presumably for this reason it must be sequestered within the heme pockets of hemoproteins, controlling its reactivity. However, under biological stress conditions, hemoproteins can release their prosthetic groups, generating “free heme”, which binds loosely to proteins or to other molecules and presumably acquires unfettered redox activity. Moreover, a growing body of evidence supports the notion that “free heme” can act in a vasoactive, pro-inflammatory and cytotoxic manner when released from a subset of these hemoproteins, such as extracellular hemoglobin, generated during hemolytic conditions. Presumably, “free heme” contributes to the pathogenesis of immune mediated inflammatory diseases associated with hemolysis. Current limitations in methodological approaches allowing a qualitative and quantitative analyzes of “free heme”, hinder the capability of determining unequivocally its pathologic effects as well as its eventual therapeutic targeting. To overcome this, we use phage display technology to generate a panel of single domain antibodies (sdAbs), namely variable light (VL), recognizing specifically “free heme”. We selected the sdAb 2H10, as being the most specific for “free heme”, characterized heme binding to this sdAb using spectroscopic techniques (UV-Visible absorption, Resonance Raman and Circular Dichroism). This revealed the presence of a 6 coordinate low spin ferric heme probably, with a cysteine as the fifth ligand and possibly a lysine or tyrosine as the sixth axial ligand. Furthermore, the secondary structure of the sdAb, composed predominantly by anti-parallel β-sheets, undergoes conformational changes to accommodate heme. We also demonstrate that these sdAb can be used to detect “free heme” in human cells using immunofluorescent techniques or flow cytometry as well as in plasma using sdAb based sandwich enzyme linked immunoabsorbant assay (ELISA).

To verify the existence of “free heme” in plasma, we used a well-established experimental model of phenylhydrazine induced acute hemolysis in mice. This assay revealed that no “free heme” is detected in plasma even though total heme levels and cellular heme uptake increased while the heme binding capacity of plasma decreased transiently. Presumably, the high buffer capacity of plasma provided by proteins or other biological molecules provides a tight regulatory mechanism for the maintenance of systemic heme homeostasis. We also demonstrated that the binding of sdAb 2H10 inhibits the heme redox activity, presumably contributing for the prevention of the deleterious effect of heme.

In summary, we demonstrate that sdAbs can be used to overcome the current limitation in the quantitative and qualitative analysis of “free heme” under hemolytic conditions or other biological conditions presumably associated with the release of heme from hemoproteins. We propose the usage of the sdAbs, namely sdAb 2H10 for therapeutically targeting of “free heme” as well as to modulate its redox activity in pathologies associated with hemolysis.

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CHAPTER 1

GENERAL INTRODUCTION

1 THE BIOLOGY OF HEME

Heme is an iron (Fe)-containing protoporphyrin IX molecule involved in vital biological processes in numerous organisms, including mammals. It is incorporated as prosthetic group of hemoproteins that have crucial roles in biological processes, such as gas storage and transport, enzymatic catalysis and regulation of gene expression, among others (Chapman et al., 1997;Poulos, 2007b).

The distribution of heme varies among different organisms, tissues and cells. In humans around 80% of heme remains in red blood cells (RBC) while 15% is found in the liver and the remaining 5% in other tissues (Zhang, 2011). Nevertheless, all mammalian cells require basal levels of heme used for the synthesis and correct function of crucial hemoproteins.

1.1 Discovery of The Heme Molecule

The circulatory system sustains blood transit through the organism and as such, is critical to maintain homeostasis. For instance, it transports nutrients (such as amino acids; aa and electrolytes), oxygen (O_2), carbon dioxide (CO_2), hormones, proteins as well as RBC and immune cells. RBCs are the most common cell type in blood, accounting for around 35-40% of the total blood volume ($5x10^6$ RBC/μm^3 of blood). The remaining volume corresponds to plasma, composed by water containing around 70 g/L proteins (*e.g.* albumin, globulins) and electrolytes (*e.g.* sodium, calcium, magnesium), glucose, hormones and other compounds as well as other cell types (Edsall, 1972b).

In the early 1840s, a pigment conferring the intense red color to the RBC was crystallized from mammals, birds and fishes (Edsall, 1972b). At around 1864, this pigment was further studied using spectrophotometry and named hemoglobin by Hoppe-Seyer (Garcia-Sanchez et al., 2013). The discovery of co-factor of hemoglobin started in 1853 by Teichmann, with the first description of the separation of the blood pigment into a crystal with a reddish-brown color and a colorless protein (Fischer and Zeile, 1929). The reddish-brown crystal substance was named heme, a molecule containing Fe, and the colorless protein was named

globin, the two assembling to form hemoglobin (Edsall, 1972a). It was only in 1929 however, that the chemical structure of heme was revealed by Fischer, who synthesized hemin, *i.e.* chloride (Cl) Fe-protoporphyrin (PP) IX molecule, and confirmed its structure, as previously proposed by Piloty and Küster (Fischer and Zeile, 1929). This discovery was awarded with the Nobel Prize in Chemistry in 1930 in recognition of "researches into the constitution of haemin and chlorophyll and especially for his synthesis of haemin" (Fischer and Zeile, 1929;Garcia-Sanchez et al., 2013).

1.2 Structure and Biochemistry of Heme

Heme is a hydrophobic metallo-compound composed by four methane-bridged pyrroles (tetrapyrrole ring) bound to a central Fe atom through their nitrogen atoms (**Fig. 1**) (Kumar and Bandyopadhyay, 2005;Tsiftsoglou et al., 2006;Poulos, 2007b). The Fe atom can acquire different oxidative states, most commonly ferrous (Fe^{2+}) with a neutral chemical charge or ferric (Fe^{3+}), positively charged and optimized for anion binding (Kumar and Bandyopadhyay, 2005). Although ferrous and ferric are the most common, other oxidation heme states from Fe^{2+} to Fe^{5+} can occur, as observed in cytochrome c oxidase or peroxidases (Ogura et al., 1996). Additionally, the planar conformation of heme can suffer small distortions within proteins, which are very common and functionally relevant (Shelnutt et al., 1998).

Heme displays a variety of subtle chemical conformational modifications, giving rise to different heme *a*, *b* and *c* variants (**Fig. 1**) (Chapman et al., 1997;Tsiftsoglou et al., 2006;Smith et al., 2010). The most common and abundant heme type in mammals is heme *b,* present in hemoglobin and myoglobin, among others (Evans and Brayer, 1990;Kakar et al., 2010a). While heme *b* displays the standard structure of Fe protoporphyrin IX, heme *a*, found in cytochrome c oxidase, has the vinyl group replaced by a 17-hydroxyethylfarnesyl group and one methyl group replaced by a formyl group (Caughey et al., 1975) (**Fig. 2**). In heme *c*, the vinyl groups are replaced by sulfhydryl groups (Bowman and Bren, 2008) (**Fig. 2**), as found in cytochrome *c* (Cytc) (Tsukihara et al., 1995). Whereas heme *c* can bind covalently to proteins, *via* two thioether bonds, heme *b* and *a* cannot, allowing their

release from hemoproteins (Allen et al., 2003). Other heme variants can be found in a variety of organisms such as bacteria, namely heme *d* (Murshudov et al., 1996), heme *d1* (Sjogren and Hajdu, 2001), heme *o* (Abramson et al., 2000), heme P460 (Igarashi et al., 1997) and siroheme (Oliveira et al., 2008) (**Fig. 2**).

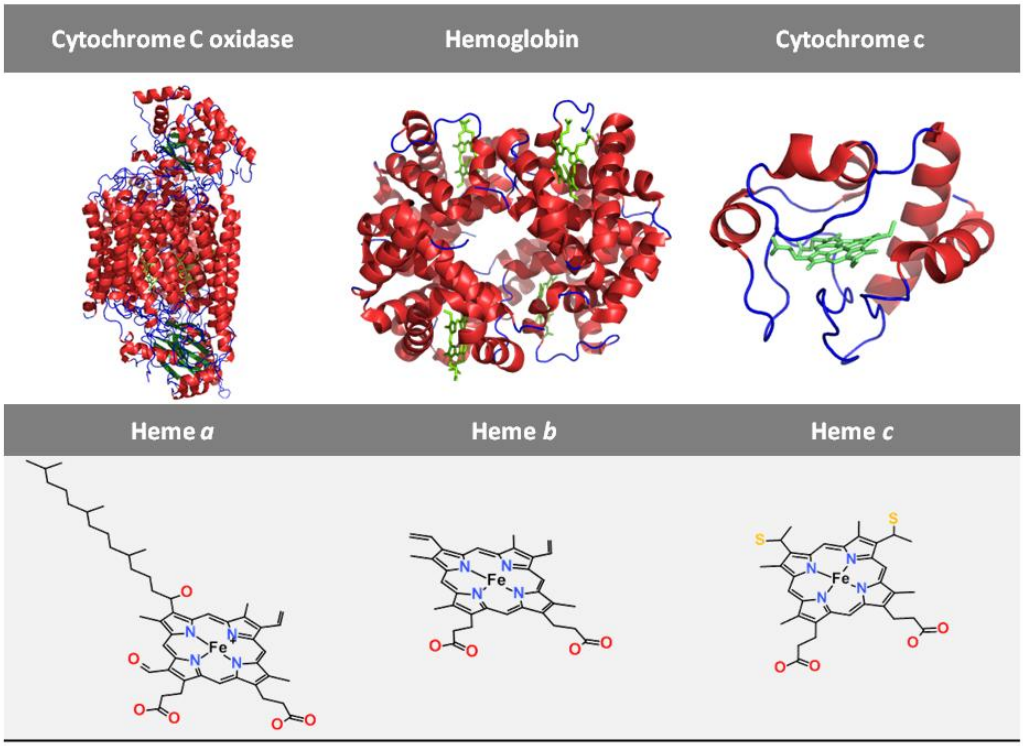
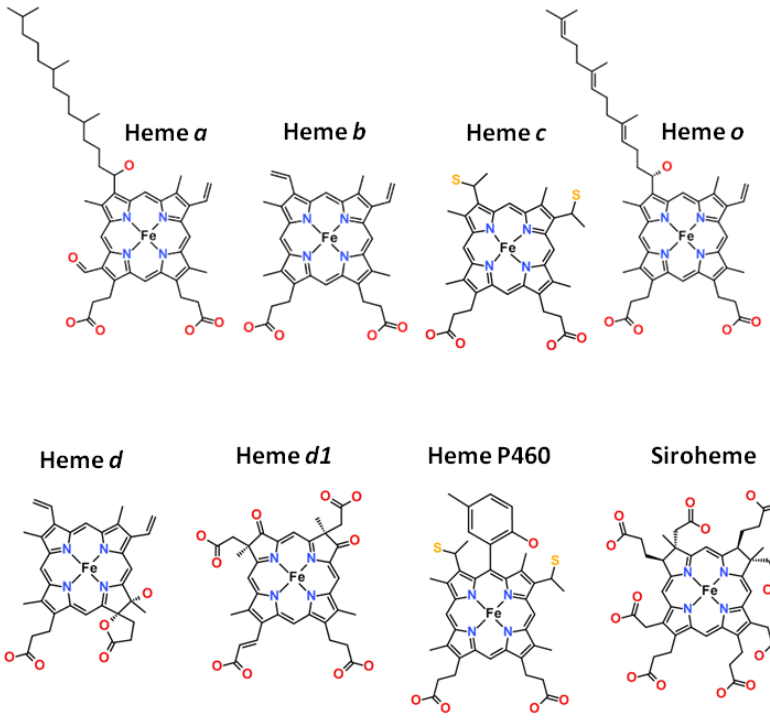


Figure 1: Hemoproteins containing the most common heme types. Crystalographic structure of bovine heart cytochrome C oxidase (PDB ID: 1OCC), human hemoglobin (PDB ID: 2W6V) and horse heart cytochrome c (PDB ID: 1HRC) (upper) and their prosthetic group, heme type a (ChEBI: 83281), heme b group (ChEBI: 55376) and heme c group (ChEBI: 60562) (lower), respectively.



Type	%*
Heme *a*	2
Heme *b*	53
Heme *c*	39
Heme *o*	1
Heme *d*	2
Heme *d1*	1
Heme P460	1
Siroheme	1
Myeloperoxidase	1

Figure 2: Structure of the different heme groups existent in hemoproteins from a variety of species. Heme types: a (ChEBI: 83281), b (ChEBI: 55376), c (ChEBI: 60562), d (ChEBI: 62802), d1(ChEBI: 61147), P460(ChEBI: modified 60562), o (ChEBI: 36301), siroheme (ChEBI: 28599) and myeloperoxidase (also known as heme m, ChEBI: 55376) are found in hemoproteins, such as cytochrome c oxidase, hemoglobin, cytochrome c, catalase hydroperoxidase II, cytochrome cd1 nitrite reductase, hydroxylamine oxidoreductase, ubiquinol oxidase, sulfite reductase and myeloperoxidase, respectively. These structures were manipulated according to their ChEBI (Chemical Entities of Biological Interest; http://www.ebi.ac.uk/chebi/), using the software Acceryls Draw. Table shows the relative abundance of the heme type amongst all hemoproteins described in the database; http://www.hemeprotein.info/detailquery.php, *i.e.* for a total of 112 different hemoproteins among several species, respectively.

1.2.1 Heme Interaction with Proteins

Heme is essential for the proper folding and function of hemoproteins (Chapman et al., 1997;Poulos, 2007b) that perform a wide range of biological functions, including transport (*e.g* hemoglobin) and storage (*e.g.* myoglobin) of diatomic gaseous molecules such as oxygen (O_2), nitric oxide (NO) and CO (Evans and Brayer,

1990;Aono, 2008;Kakar et al., 2010b). Some hemoproteins are endowed with enzymatic activity (*e.g.* peroxidases, catalase and synthases) (Reid et al., 1981;Crane et al., 1997;Zederbauer et al., 2007b;Capitanio et al., 2011) and are involved in key metabolic processes (*e.g.* cytochromes P-450, peroxidases) (Bushnell et al., 1990;Guengerich, 2001). These include the respiratory electron transport chain in the mitochondria (*e.g.* cytochromes c, cytochrome c oxidase) (Gray and Winkler, 1996;Capitanio et al., 2011), gene transcription (*e.g.* heme binding to the transcription regulatory protein basic leucine zipper transcription factor 1, BACH1) (Youn et al., 2006;Hira et al., 2007), micro-RNA processing (*e.g* RNA-binding protein DiGeorge critical region-8, DGCR8) (Weitz et al., 2014), regulation of circadian rhythms (*e.g.* Neuronal PAS domain-containing protein 2, NPAS2)(Kaasik and Lee, 2004) and signal transduction pathways (*e.g.*BACH1) (Zenke-Kawasaki et al., 2007) or ion-channel functions (*e.g*. sodium channel) (Horrigan et al., 2005;Wang et al., 2009).

1.2.1.1 Heme Pocket

The “heme pockets” of hemoproteins (**Fig. 1 and 3; Table 1**) are characterized by a high abundance of aromatic amino acids (aa), *i.e.* as phenylalanine (Phe, F), tyrosine (Tyr, Y) or tryptophan (Trp, W) and by few non-charged aa (Li et al., 2011), conferring the hydrophobicity required to stabilize heme and its binding to protein matrix. Furthermore, hemoproteins contain frequently five highly conserved aa in their heme pockets, including histidine (His, H), methionine (Met, M), cysteine (Cys, C), Tyr and lysine (Lys, K) that can act as axial Fe-heme ligands (Li et al., 2011). Beside these, other aa can bind to Fe-heme, including proline (Pro, P) through its N-terminus amino group (Lanzilotta et al., 2000;Clark et al., 2004) or asparagine (Asp, N) *via* the ε-amine group (Leys et al., 2000). More recently, it has been proposed that threonine (Thr, T) can also support heme binding to proteins (Torda et al., 2013). Of these aa residues, His is the one found most frequently in the heme pocket of hemoproteins containing heme *c* or *b,* while Cys usually promotes binding to heme *b,* and Met to heme *c* (**Table 1**) (Fufezan et al., 2008;Li et al., 2011). Other hydrophobic aa such as leucine (Leu, L), isoleucine (Ile, I) and valine (Val, V) can

also establish interactions with the heme porphyrin, whereas arginine (Arg, R) and other positively charged aa can interact with the negatively charged heme propionate groups (**Fig. 3)** (Schneider et al., 2007).

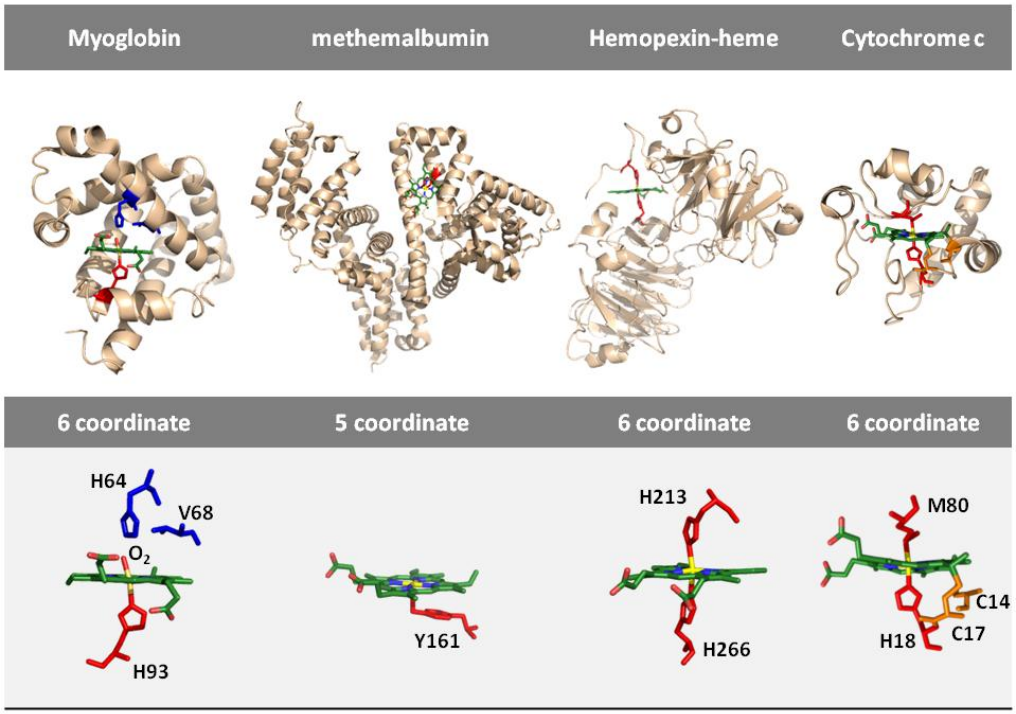


Figure 3: Hemoproteins with their respective heme pockets. Structure of human sperm whale oxymyoglobin (PDB ID: 1MBN), methemalbumin (PDB ID: 1N5U), rabbit heme scavenger hemopexin bound to heme (PDB ID: 1QJS) and horse heart cytochrome c (PDB ID: 1HRC) (upper) with their heme group highlighted. Detail view of heme group of the corresponding hemoproteins (lower). The heme-Fe in oxymyoglobin is a six coordinate (6c) low spin (LS) with a histidine (H93) and a molecule of oxygen (O_2) axial ligands. Upon oxygen dissociation, myoglobin acquires a five coordinate (5c) high spin (HS) heme state. Heme in albumin is a 5cHS with a tyrosine (Y161) amino acid bound to Fe. In hemopexin, heme has two histidine (H213 and H266) coordinated to its Fe atom and therefore a 6cLS state. Unlike in heme b type hemoproteins, heme in cytochrome c is covalently attached to the protein through two cysteines (C14 and C17), as thus defined as heme c; its Fe is coordinated with a proximal Histidine (H18) and a distal methionine (M80), forming a 6c LS state.

Table 1: Selected representatives of mammalian hemoproteins and heme transient binding proteins. Proteins were classified according to their function and the number and type of heme were indicated as well as the heme axial ligands.

Function	Representative Proteins	Heme Number/ Type	Axial ligand(s)	Reference
Electron transport	Cytochrome c	1/ *c*-type	His/Met	(Mirkin et al., 2008)
	Cytochrome b5	1/ *b*-type	His/ His	(Wirtz et al., 2000)
	Cytochrome c oxidase (complex IV)	2/ *a*-type	His His/His	(Tsukihara et al., 1995)
	Cytochrome c reductase/ cytochrome bc_1	2/ *b*-type 1/ *c*-type	His/His His/His His/Met	(Xia et al., 1997;Zhang et al., 1998)
Gas binders and carriers	Hemoglobin	4 / *b*-type	His	(Park et al., 2006)
	Myoglobin	1 / *b*-type	His	(Evans and Brayer, 1988;Vojtechovsky et al., 1999)
Catalytic activity - enzyme	Catalase I	1/ *b*-type	Tyr	(Putnam et al., 2000)
	Cytochrome P450 (2D6)	1/ *b*-type	Cys	(Rowland et al., 2006)
	Indoleamine 2,3-dioxygenase	1/ *b*-type	His/ligand	(Sugimoto et al., 2006)
	NO synthase	2/ *b*-type	Cys	(Crane et al., 1997)
	Cystathione β-synthase	1/ *b*-type	Cys/His	(Meier et al., 2001)
	Tryptophan 2,3 dioxygenase	4/ *b*-type	His	(Zhang et al., 2007)
Heme degradation	Heme Oxygenase	1/ *b*-type	His/ H_2O	(Lad et al., 2003)
Heme binding and transport	Hemopexin	1/ *b*-type	His/His	(Paoli et al., 1999)
	Albumin	1/ *b*-type	Tyr	(Wardell et al., 2002)
	α_1-microglobulin	1/*b*-type	His/Cys	(Allhorn et al., 2003;Larsson et al., 2004;Meining and Skerra, 2012)
Heme-sensing /heme regulation	NPAS2	2/*b*-type	His/Cys or His/His His/?	(Dioum et al., 2002a;Uchida et al., 2005)
	Bach 1	5/*b*-type	4x Cys 1xCys/His	(Hira et al., 2007)
	Iron regulatory protein 2	2/*b*-type	Cys/? His/His	(Jeong et al., 2004a;Jeong et al., 2004b;Ishikawa et al., 2011)
	Soluble guanylyl cyclase	1/ *b*-type	His	(Pellicena et al., 2004;Martin et al., 2005;Ma et al., 2007)

The axial coordination of Fe in heme is directly correlated with protein function. Fe establishes primarily four bonds with the pyrrole nitrogens of protoporphyrin IX and one or two additional bounds with aa of the heme pocket (*i.e.* the axial ligands) constituting the five (5c) or six coordinate (6c) heme, respectively (**Fig. 3)**. Usually, in gas transporter proteins such as myoglobin and hemoglobin, heme is in a 5c state with His as the fifth axial ligand while the remaining binding site is vacant for the binding of small molecules such as oxygen (Vojtechovsky et al., 1999;Park et al., 2006). In other proteins, namely enzymes with a 5c, one of the axial ligands can be either His or Tyr while the sixth axial is ligand-free and available for substrate binding (Reid et al., 1981;Rowland et al., 2006). Cytochromes involved in electron transfer reactions typically display six coordinated heme, in which both axial ligands are usually occupied by a bi-His or by His-Met (Mirkin et al., 2008).

In some hemoproteins, heme performs catalysis in which the Fe and the protoporphyrin IX ring can undergo chemical and/or electronic modifications, as demonstrated for peroxidases, as well as cytochrome P450 and cytochrome c oxidase (Zederbauer et al., 2007b). Hydrogen peroxide (H_2O_2) can be used as substrate in oxido-reductive reactions catalyzed by heme, undergoing conformational alterations at the level of the protoporphyrin IX ring as well as in the Fe oxidation state. This is followed by oxidation of the second substrate, such as indoles, phenols, aromatic amines, lignin, manganese (Mn^{2+}) ions, halide ions or proteins (Badyal et al., 2006;Zederbauer et al., 2007a). In other hemoproteins however, heme does not perform catalysis and the Fe redox state or protoporphyrin conformation are not altered (Poulos, 2007a). This is the case for hemoproteins that bind, transport or store gaseous molecules, such as hemoglobin, myoglobin, guanylate cyclase and NPAS2 (Dioum et al., 2002b;Poulos, 2007b). Binding of NO or CO to these hemoproteins modulates their biological activity, as illustrated by the increased production of cyclic guanosine monophosphate (cGMP) when NO or CO bind to the heme group of guanylate cyclase (Ma et al., 2007), regulation of O_2 and carbon dioxide (CO_2) transport in the case of hemoglobin or regulation of gene transcription in the case of NPAS2 (Dioum et al., 2002b). Alternatively, heme may

also bind proteins to regulate their biologic function, as it is the case for heme driven inhibition of the transcriptional repressor BACH1 (Hira et al., 2007).

1.2.1.2 Heme Binding Motifs

Hemoproteins possess characteristic sequences of aa, contained inside heme pockets, referred as heme-binding motifs (HBM) or heme regulatory motifs (HRM). There are two HBM that bind heme heme *c* covalently, namely CXXCH and CXXCK in which "X" refers to any aa (Hartshorne et al., 2007;Fufezan et al., 2008;Smith et al., 2010;Li et al., 2011). Other HBM, such as CXXXCH and CXXXXCH, present in multi-hemic cytochromes also bind heme *c* (Aragao et al., 2003). The AXXCH and FXXCH HBM bind heme *c* covalently through a single thioether bond (Ginger et al., 2012). In myeloperoxidases, heme *c* is bound covalently by two thioether bonds as well as by a unique methionyl-sulfonium bond involving a Met (Kooter et al., 1999). In cytochrome P460, heme *c* is also bound covalently through a CXXCH motif by an additional ligation to other aa, namely a Lys in cytochrome P460 (Pearson et al., 2007) or a His in hydroxylamine oxidoreductase (Igarashi et al., 1997).

Heme *a*, *b*, *d* (Murshudov et al., 1996), *d1* (Sjogren and Hajdu, 2001), *o* (Abramson et al., 2000) and siroheme (Oliveira et al., 2008) (**Fig. 2**) create non-covalent hydrophobic and electrostatic interactions with hemoproteins. For instance, heme *b* is mainly associated to a HBM with the aa GX[H/R]XC[P/L/ A/V]G, *i.e.* Glycine (Gly, G); X; His or Arg; X; Cys; Pro or Leu or Alanine (Ala, A) or Val and Gly, where X can be any aa (Li et al., 2011). Another related FXXGXXCXG motif, *i.e* Phe; X; X; Gly; X; X; Cys; X and Gly, was described in the mammalian cytochrome P450 as well as in plant and bacterial hemoproteins that bind heme *b* (Li et al., 2011). There is yet another common HBM composed by two aa only, Cys and Pro (*i.e.* CP) that can accommodate heme *b* (Ishikawa et al., 2005;Yang et al., 2008;Li et al., 2011). More recently, similar motifs for the binding of heme *b* were proposed, *i.e.* the Cys and aspartic acid (Asp, D) (*i.e.* CD) and the Cys and Serine (Ser; S) (*i.e.* CS) motifs (Yang et al., 2010a;Westberg et al., 2011).

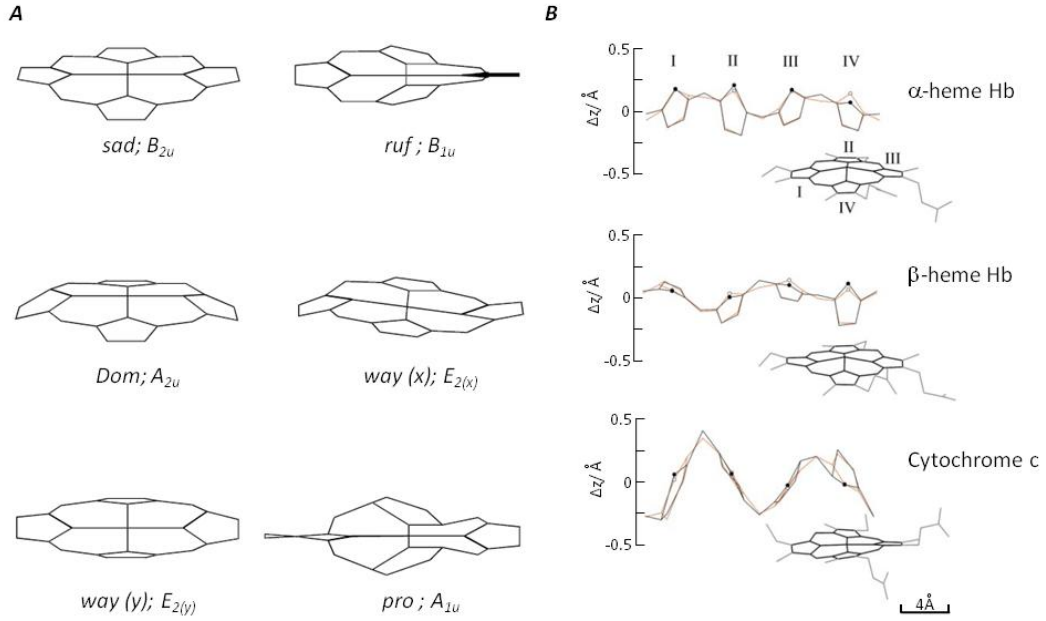


Figure 4: Non-planar distortions in the porphyrin macrocycle in different hemoproteins. Although heme is usually represented as a planar structure; it acquires different non-planar distortions in proteins. A. There are six lowest frequency out-of-plane normal coordinate deformations of the porphyrin macrocycle used to decomposed the non planar distortions of heme in proteins. These are referred to as saddling (sad, B2u), ruffling (ruf, B1u), doming (dom, A2u), waving [wav(x), wav(y); Eg], and propellering (pro,A1u). B. Non-planar distortion of the heme porphyrin macrocycle are dependent of the heme pocket of the hemoproteins, as exemplified for heme in α- or β- of deoxyhemoglobin A subunits and for the yeast isoenzyme-1 of cytochrome c (*Figures obtained from (Shelnutt et al., 1998)*).

1.2.1.3 Non-planar Heme Structure

Heme is classically represented in its planar conformation, which is the most favorable thermodynamically (**Fig. 1 and 2**). However, in the heme pocket of proteins, heme is exposed to several forces exerted by the surrounding aa, which induce distortions in the porphyrin structure (Shelnutt et al., 1998). This has been observed in several proteins with similar biological functions and in different organisms (**Fig. 4**), suggesting that alterations to the nonplanar structures of heme

modulate hemoproteins function (Shelnutt et al., 1998). To understand this observation, Shelnutt and co-workers developed a computational procedure named “normal-coordinate structural decomposition method”, that allows determining and comparing non-planar distortions of heme, as assessed in crystal structures of hemoproteins. These include six lowest frequency out-of-plane normal coordinate deformations, namely: *saddling* (*sad*, B2u), *ruffling* (*ruf*, B1u), *doming* (*dom*, A2u), *waving* [*wav*(*x*), *wav*(*y*); Eg], and *propellering* (*pro*,A1u) (**Fig. 4**) (Jentzen et al., 1998;Shelnutt et al., 1998). The biggest heme distortions were found in type-c cytochromes and peroxidases that present mainly saddled distortions, while in myoglobin (type-b), heme was predominantly found in a domed conformation (Jentzen et al., 1998;Shelnutt et al., 1998).

2 HEME BIOSYNTHESIS

Heme biosynthesis is an essential metabolic pathway in most eukaryotic and prokaryotic cells (Layer et al., 2010). The synthesis of both hemoproteins and heme are coordinated so that heme binding to hemoproteins provides the correct protein conformation and biological function. Biosynthesis of heme has been intensively studied in eukaryotes, comprising eight steps, each involving different enzymes, expressed in the mitochondria and in the cytoplasm (**Fig. 5**) (Layer et al., 2010;Hamza and Dailey, 2012). The first step consists in the condensation of Gly and succinyl-CoA into δ-aminolevulinic acid (ALA) and CO_2, which is catalyzed by the mitochondrial δ-aminolevulinate synthase (ALAS) (E.C. 2.3.1.37). This enzyme acts as the rate-limiting step of the pathway and exists in mammals in two isoforms: ALAS1 (or ALAS-N) and ALAS2 (or ALAS-E). ALAS1 is encoded by the house-keeping gene *ALAS1* (chromosome 3), which is expressed in all tissues, including cells of the erythroid lineage, and provides the basal levels of heme necessary for maintenance of cellular homeostasis. ALAS2 is encoded by the *ALAS2* gene (chromosome X) and is expressed only during the differentiation of erythroid cells, when an increase in the amounts of heme is required to support hemoglobin synthesis (Kramer et al., 2000;Harigae et al., 2003).

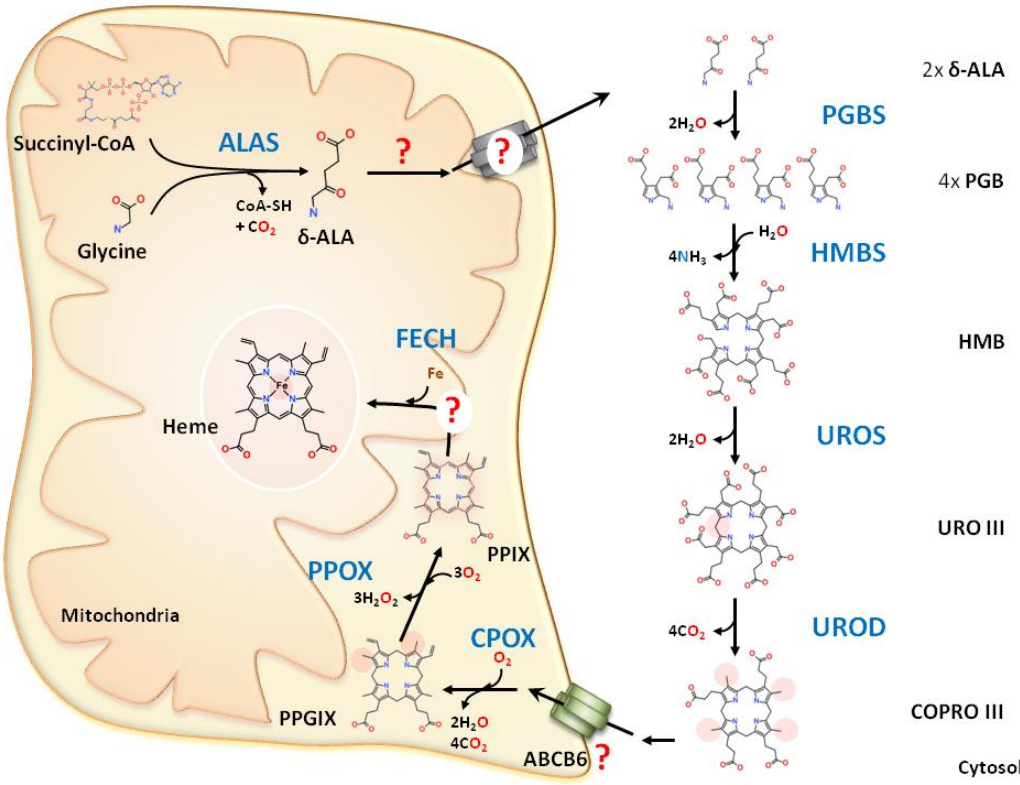


Figure 5: Heme biosynthesis. The first step of heme biosynthesis occurs in the mitochondrial matrix with the condensation of glycine and succinyl-CoA by the mitochondrial δ-aminolevulinate synthase (ALAS) to generate δ-aminolevulinic acid (ALA). ALA is then translocated to the cytosol by unknown mechanism where the next four steps of heme biosynthesis occur. In the cytosol, two molecules of ALA react with pyridoxal phosphate to form a porphobilinogen (PBG) through the catalyst porphobilinogen synthase (PGBS). Four molecules of PBG are condensed by hydroxylmethylbilane synthase (HMBS), generating a molecule of hydroxymethylbilane (HMB) that is converted into uroporphyrinogen III (URO III) by uroporphyrinogen III co-synthase or isomerase (UROS). The last step in the cytosol is the conversion of decarboxylation of URO III into coproporphyrinogen III (COPRO III) by uroporphyrinogen III decarboxylase (UROD). COPRO III is then translocated into the mitochondrial intermembranal space by a proposed transporter, ABCB6 and then by oxidative decarboxylation it is converted into protoporphyrinogen IX (PPG IX) by the coproporphyrinogen oxidase (CPOX). This is followed by PPG IX oxidation by protoporphyrin oxidase (PPOX) into protoporphyrin IX (PPIX) that is translocated to the mitochondrial matrix, for ferrous iron cation (Fe^{2+}) incorporation by the enzyme ferrochelatase (FECH) into the macrocycle of PPIX to form protoheme IX (heme).

Both ALAS proteins harbor two putative HBM in the mitochondrial targeting peptide at the N-termini, and a third HBM near the N-terminus of the mature protein (Dailey et al., 2005). Heme binding to the HBM of the immature protein presumably blocks mitochondrial import and consequently heme synthesis, thus avoiding the accumulation of intracellular heme that would be otherwise deleterious (Munakata et al., 2004). This level of regulation occurs only on the ALAS1 and not on the ALAS2 isoform (Munakata et al., 2004). *ALAS1* gene expression is down-regulated by heme itself, while *ALAS2* expression is controlled at the transcriptional level by different erythroid specific factors, such as GATA binding protein 1 (globin transcription factor 1; GATA1) (Surinya et al., 1997).

The following four steps in heme biosynthesis occur after ALA translocates into the cytosol, by a still unknown mechanism. Two molecules of ALA react with pyridoxal phosphate to form a porphobilinogen through the activity of catalyst porphobilinogen synthase (E.C. 4.2.4.24) (Bollivar et al., 2004). Then, four porphobilinogen molecules are condensed by hydroxylmethylbilane synthase (E.C. 2.5.1.61) to generate hydroxymethylbilane (Gill et al., 2009). In the absence of the next enzyme of the heme pathway, *i.e.* uroporphyrinogen III co-synthase or isomerase (E.C. 4.2.1.75), the chemically unstable hydroxymethylbilane can spontaneously react to generate uroporphyrinogen I, an end product that cannot lead to heme formation. The uroporphyrinogen III co-synthase catalyzes the enzymatic reaction in which four molecules of hydroxylmethylbilane are converted into uroporphyrinogen III (Schubert et al., 2008). The last reaction occurring in the cytosol consists in a decarboxylation of uroporphyrinogen III to form coproporphyrinogen III by uroporphyrinogen III decarboxylase (E.C. 4.1.1.37) (Phillips et al., 1997). Later, coproporphyrinogen III is transported to the mitochondrial intermembranal space, presumably by the ATP-binding cassette sub-family B member 6 (ABCB6) (Krishnamurthy et al., 2006), where the coproporphyrinogen oxidase (E.C. 1.3.3.3) catalyzes the oxidative decarboxylation of coproporphyrinogen III into protoporphyrinogen IX, that is subsequently oxidized to protoporphyrin IX (PPIX) by protoporphyrin oxidase (E.C. 1.3.3.4), producing three moles of hydrogen peroxide (Sorianello and Mazzetti, 2000). The final step

consists at the incorporation of a ferrous Fe cation (Fe^{2+}) into the macrocycle of PPIX to form protoheme IX (heme), by the enzyme ferrochelatase (FECH) (E.C. 4.99.1.1) in the mitochondrial matrix (Wu et al., 2001). The *de novo* synthesized heme is finally transported to the cytosol where it can either be incorporated into apoproteins to form hemoproteins or be directly involved in other biological functions (*e.g.* transcription, translation, cellular differentiation).

3 HEME TRANSPORTERS

Heme transport is tightly regulated so that heme is delivered to different compartments where it is used in diverse biological processes. The hydrophobic nature of heme determines its insolubility in aqueous solutions, making it unlikely that heme could be transported as a free molecule and suggesting the existence of heme binding partners that assist its transport from one cell compartment to another. Additionally, its hydrophobic nature should allow heme diffusion through cellular membranes, although this was shown to occur rather slowly, partially due to the hydrophilicity conferred by the propionate groups of heme (Light and Olson, 1990). This dual nature of heme and its limitations to diffuse freely through membranes imposes the need for heme transporters, and several have been described (**Fig. 6; Table 2**)**.** Considering that "free heme" is cytotoxic (Gozzelino et al., 2010;Larsen et al., 2010;Gozzelino and Soares, 2011) "carriers" and/or chaperone proteins transport heme to specific apoproteins or to be degraded in different cell compartments and/or tissues.

3.1 Heme Importers

Cellular heme transporters include Heme-Responsive Gene-1 (HRG-1), heme carrier protein 1 (HCP-1) and Feline Leukemia Virus Subgroup C Receptor 2 (FLVCR2). HRG-1 was discovered in the roundworm *Caenorhabditis elegans*, a heme auxotroph that acquires heme exclusively from the environment (Rajagopal et al., 2008). HRG-1 has orthologs in several organisms such as *Leishmania*, zebrafish and mammals (Rajagopal et al., 2008;White et al., 2013).

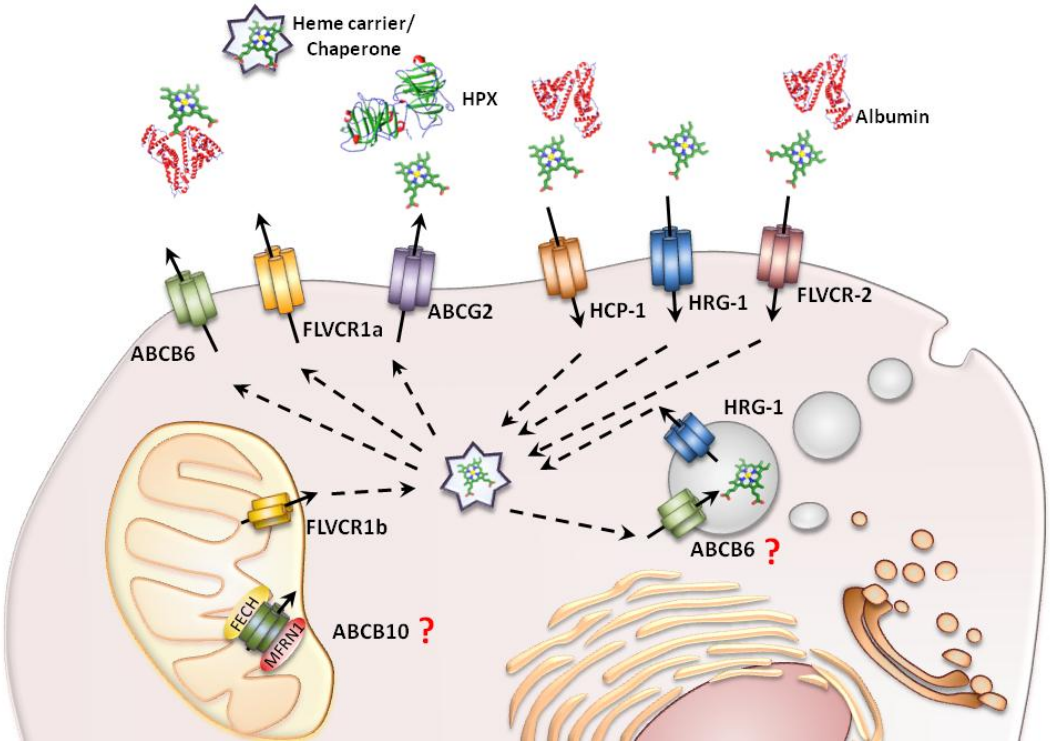


Figure 6: Extracellular and intracellular heme transport. As “free heme” is highly cytotoxic, intracellular heme is maintained bound to a heme carrier/chaperone, providing a support for heme transfer between the different compartments and transporters. Intracellular heme transport is mediated by at least three importers, *i.e.* Haem carrier protein 1 (HCP-1), feline leukemia virus subgroup C cellular receptor 2 (FLVCR2) and Heme-responsive gene 1 protein (HRG-1) and three cellular membranar exporters, *i.e.* ATP-binding cassette, sub-family B 6 (ABCB6), feline leukemia virus subgroup C cellular receptor 1a (FLVCR1a) and ATP-binding cassette sub-family G member 2 (ABCG2). HCP-1 has been described to be responsible for heme uptake in the intestine and FLVCR2 from a wide range of mammalian cells. HRG-1 was initially described to export heme from the lysosome/endosome compartment; however, HRG-1 can travel to the plasma membrane and import heme (Rajagopal et al., 2008). ABCB6 is localized in the plasma membrane as well as in the lysosome/endosome compartment (Kiss et al., 2012). FLVCR1b is responsible for heme export from the mitochondria into the cytosol. ABCB10 forms a complex with MFRN1 and FECH, probably acting as a mitochondrial exporter.

The mammalian HRG-1 ortholog is highly expressed in the brain, heart, kidney and muscle and to a lesser extent in liver, lung, placenta and intestine (Rajagopal et al.,

2008). HRG-1 is localized in the membranes of endosomes and lysosomes and mediates heme transport from these acidic compartments into the cytoplasm (**Fig. 6**). More recently, HRG-1 was identified in the plasma membranes and lysosomes of non-polarized human epithelial type 2 (Hep2) cells as well as coupled to the vacuolar ATPase (adenylpyrophosphatase; EC 3.6.1.3) proton pump, which induces endosomal acidification, increasing HRG-1 affinity towards heme.

Table 2: Putative heme and/or porphyrin transporters. The transporters were classified according to their functions and then localization and expression.

Transporters	Protein	Localization	Expression	Reference
Importers	HRG-1	PM of endosomes/ lysosomes	brain, heart, kidney, skeletal muscle, liver, lung, placenta, intestine	(Rajagopal et al., 2008;White et al., 2013)
	HCP-1	PM	duodenal and jejunal enterocytes and liver	(Shayeghi et al., 2005;Qiu et al., 2006)
	FLVCR2	PM	Multiple tissues: liver, fetal liver, brain, lung, kidney ,placenta	(Quigley et al., 2004;Duffy et al., 2010)
Exporters	FLVCR1a	PM	hematopoietic tissues (PBL, fetal liver, spleen, Lymph node), pancreas, kidney, placenta	(Tailor et al., 1999;Vinchi et al., 2014)
	FLVCR1b	MIM	progenitors of the hematopoetic cells	(Chiabrando et al., 2012)
	ABCG2 (BCP)	PM	Multiple tissues and highly in stem cells	(Zhou et al., 2001;Krishnamurthy and Schuetz, 2005)
	ABCB6	PM of endosomal/ lysosomal	erythrocyte membrane, fetal liver	(Krishnamurthy et al., 2007;Kiss et al., 2012)
	ABCB10	MIM	Multiple tissues: fetal liver, kidney, BM and erythrocytes	(Shirihai et al., 2000;Chen et al., 2010)

Plasma membrane (PM); Mitochondrial outer membrane (MOM); mitochondrial inner membrane (MIM); Bone Marrow (BM); blood-brain barrier (BBB); peripheral blood lymphocytes(PBL)

HCP-1 is a low affinity and non-exclusive heme cellular importer (*e.g.* it can also translocate folate), expressed mainly in the liver and in duodenum enterocytes and, to a lesser extent in jejunum enterocytes (**Fig. 6**). HCP-1 is responsible for heme absorption from food (Shayeghi et al., 2005;Qiu et al., 2006;Le Blanc et al., 2012).

FLVCR2 is a heme cellular importer expressed in several mammalian non-hematopoietic tissues including brain, placenta, lung, liver, and kidney as well as in hematopoietic tissues, including fetal liver, spleen, lymph nodes, thymus, leukocytes, and bone marrow (Duffy et al., 2010)(**Fig.** the *FLVCR2* **6**). Mutations in gene were linked to Fowler syndrome, a vascular disorder of the brain, but the association of its cellular function as a heme cellular importer with the disease still remains to be elucidated (Duffy et al., 2010).

3.2 Heme Exporters

After the last step of *de novo* heme synthesis, newly generated heme must be exported from the mitochondria so that it can be incorporated into apoproteins, generating a variety of hemoproteins. Two heme mitochondrial exporters have been described, namely, the Feline Leukemia Virus Subgroup C Receptor 1 (FLVCR1) (Quigley et al., 2004) and the ATP-binding cassette transporter G2 (ABCG2)(Krishnamurthy and Schuetz, 2005).

In mammals, FLVCR1 exists in two isoforms, FLVCR1a (full-length; ~60 kDa) (Quigley et al., 2000) and FLVCR1b (shorter protein; ~28 kDa) (Chiabrando et al., 2012). FLVCR1a is responsible for heme cellular export, while FLVCR1b exports heme from the mitochondria (**Fig. 6**). FLVCR1a is expressed predominantly by hematopoietic cells, including peripheral blood lymphocytes, fetal liver, as well as leukocytes in the spleen and lymph nodes. It is also expressed in cells from non-hematopoietic tissues, including in the pancreas, kidneys and placenta (Tailor et al., 1999). FLVCR1b on the other hand, is mainly expressed in the brain, heart, skeletal muscle, bone marrow, and spleen. Interestingly, heme cellular export is increased in the presence of secreted heme binding proteins such as hemopexin and albumin, suggesting that these scavengers uptake heme delivered by FLVCR1, presumably

via a direct interaction between the transporter and the scavenger (Yang et al., 2010b). The two FLVCR1 isoforms seem to have different functions. FLVCR1b is essential for erythroid differentiation, while FLVCR1a is required for intracellular heme homeostasis among different cells types, with an important impact on heme synthesis and consequently, on hemoprotein function (Chiabrando et al., 2012).

The FLVCR1 transporter is required for erythroid maturation (Quigley et al., 2004), a notion supported by the fact that FLVCR1-null mice lack mature erythrocytes, as they develop severe macrocytic anemia and proerythroblast apoptosis (Quigley et al., 2004). This is not observed in humans carrying point mutations in this transporter, which presumably lead to alterations in its transmembrane domains (Yanatori et al., 2012), but do not lead to overt defect in erythropoiesis (Yanatori et al., 2012). This questions whether FLVCR1 is essential for heme transport and erythropoiesis in humans.

ABCG2 is another heme cellular exporter also known as breast cancer resistance protein (BCP), which was discovered based on its role in conferring drug resistance of tumor cells (Doyle et al., 1998;Miyake et al., 1999). ABCG2 is not specific for heme, as it can bind other protoporphyrins (PP) (*e.g.* Zinc PP, Cobalt PP, empty PP), preventing their accumulation within cells and contributing to PP homeostasis (Krishnamurthy et al., 2004;Desuzinges-Mandon et al., 2010). Even though ABCG2 binds to a wide range of PP, its highest binding affinity is for heme (Desuzinges-Mandon et al., 2010). Similarly to FLVCR1, it delivers heme to heme-binding proteins such as albumin (Desuzinges-Mandon et al., 2010). ABCG2 is expressed in a wide variety of cells, including hematopoetic stem cells, being particularly expressed at high levels in the early stages of hematopoiesis (Zhou et al., 2001), contrarily to FLVCR1 that is mainly expressed during erythropoiesis (Quigley et al., 2004).

Under certain pathologic conditions, heme can accumulate in cells to a level that becomes cytotoxic, causing cellular, tissue and eventually organ damage. When this occurs, heme must be exported from cells, as to avoid cytotoxicity. For instance, phagocytosis of senescent RBC by hemophagocytic macrophages (Møs) is followed by heme release from hemoglobin (Bratosin et al., 1998) and transit

from phagosomes into the cytoplasm where it is degraded by heme oxygenase 1 (HO-1), a heme catabolizing enzyme anchored in endoplasmic reticulum (White et al., 2013;Yuan et al., 2013).

Intracellular heme transport is controlled by the ATP-binding cassette sub-family B member 6 (ABCB6), localized in the mitochondrial outer membrane, which was first described as an Fe transporter and only later as a heme transporter (Krishnamurthy et al., 2006) (**Fig. 5**). ABCB6 is also present in the endosomal/lysosomal compartments (**Fig. 6**) and in the plasma membrane of mature erythrocytes and hepatocellular carcinoma cells (Helias et al., 2012;Kiss et al., 2012). Absence of ABCB6 however does not interfere with heme biosynthesis nor does it affects erythropoiesis (Helias et al., 2012;Kiss et al., 2012).

ATP-binding cassette sub-family B member 10 (ABCB10) (also known as ABC-me) is a putative heme transporter localized in the inner mitochondrial membrane, presumably forming a stable complex with mitoferritin 1 (MFRN1) and ferrochelatase (**Fig. 6**) (Chen et al., 2010). This ABC transporter is expressed in several tissues, but is mainly found in fetal liver cells, kidney, adult bone marrow, and mature erythrocytes (Shirihai et al., 2000). ABCB10-null mice have impaired primitive erythropoiesis and lack of hemoglobinized cells, leading to embryonic lethality at midgestation (Hyde et al., 2012;Yamamoto et al., 2014). Moreover, these mice have defects in heme biosynthesis with concomitant accumulation of Fe and protoporphyrin IX in the mitochondria (Yamamoto et al., 2014). This suggests that the ABC transporter is essential for heme biosynthesis and erythropoiesis, presumably through heme transport from the mitochondria to cytosol (Hyde et al., 2012;Yamamoto et al., 2014). More recently however, it has been argued that ABCB10 role in the early stages of heme biosynthesis relies on promoting not heme but ALA export from the mitochondria (Bayeva et al., 2013), at odds with a putative role of this ABC transporter as a heme transport (Hyde et al., 2012;Yamamoto et al., 2014). As such, further studies are required to assess whether ABCB10 is a genuine heme transporter.

4 HEME BINDING PROTEINS/CHAPERONES

The cytotoxic nature of “free heme” imposes that its intra- and inter-cellular as well as inter-tissue transport are tightly regulated (Korolnek and Hamza, 2014). This is achieved not only by heme transporters (*see* **section 3**) but also by heme binding proteins and/or chaperones that assist heme transporters by delivering and/or acquiring heme. These proteins bind “free heme” transiently and deliver it to other proteins or cells for degradation.

Several proteins have been identified as heme binding proteins/chaperones (**Fig. 7; Table 3**). Chaperone proteins play two major roles. On one hand they allow correct folding and assembly of nascent proteins into their functional active tertiary structure. On the other hand, they act upon stress allowing proteins to return to their initial conformation. In the early nineties, Robert Hausinger suggested that chaperones might also be responsible for the incorporation of metals, heme and heme analogs into metalloproteins (Hausinger, 1990). Nowadays, several chaperones have been described to have such function, *e.g*. the heme chaperone CcmE (cytochrome c maturation E) from the system I cytochrome c maturation (ccm) pathway found in bacteria, archaea and plants (Verissimo et al., 2013). In plants, these chaperones are expressed in the mitochondria and promote covalent heme binding to cytochrome c (Cytc). While the existence of such chaperones in mammals remains to be established, mammals have a variety of proteins that might act as heme binding proteins/chaperones, including intracellular proteins such as glutathione S-Transferase B (GST) (Caccuri et al., 1990), fatty acid binding protein (FABP) (Stewart et al., 1996), heme-binding protein 23 kDa (HBP23)/peroxiredoxin (Prx I) (Iwahara et al., 1995), heme-binding protein 22 (p22HBP) (Taketani et al., 1998;Micaelo et al., 2010), SOUL from the same family (Sato et al., 2004), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Chakravarti et al., 2010;Hannibal et al., 2012) heat-shock protein 90 (HSP90)(Ghosh et al., 2011) and biliverdin reductase (BVR) (Tudor et al., 2008).

Table 3: Described heme scavengers. The proteins were classified according to their localization and the heme binding affinity was also indicated.

Localization	Heme scavengers	Heme binding site/ affinity	Expression	Reference
Intracellular	GST	2x 10^{-6}; 10^{-8} M	Multiple tissues	(Vander Jagt et al., 1985;Caccuri et al., 1990)
	HBP24	1x 10^{-8} M	Multiple tissues	(Iwahara et al., 1995;Hirotsu et al., 1999)
	p22HBP	10^{-6} to 10^{-7} M	Multiple tissues; higher in liver/hematopoietic tissues	(Jacob Blackmon et al., 2002;Dias et al., 2006)
	SOUL	10^{-9} M	retina and pineal gland	(Zylka and Reppert, 1999;Sato et al., 2004)
	FABP	10^{-7} M (Fe^{3+}) or 10^{-8} M (Fe^{2+})	Multiple tissues; higher in liver,heart	(Vincent and Muller-Eberhard, 1985;Borchers and Spener, 1993;Epstein et al., 1994;Storch and Thumser, 2000)
	GAPDH	10^{-8} M	Multiple tissues, higher in muscle, heart, brain	(Chakravarti et al., 2010;Tristan et al., 2011;Ghosh and Stuehr, 2012)
	BVR	10^{-7} M	Multiple tissues	(Tudor et al., 2008)
Extracellular (secreted proteins)	Haptoglobin	10^{-12} M	Primarily hepatocytes; Mø / neutrophils…	(Wejman et al., 1984a;b;Okuda et al., 1992;Okazaki et al., 1997;Kristiansen et al., 2001;Thomsen et al., 2013;Mollan et al., 2014)
	Hemopexin	$<10^{-12}$M	Primarily hepatocytes; others nervous system, skeletal muscle, kidney	(Paoli et al., 1999;Shipulina et al., 2000;Tolosano et al., 2010)
	Albumin	10^{-8} M	Primarily hepatocytes; others lymph gland, skeletal muscle, mammary gland	(Adams and Berman, 1980;Margarson and Soni, 1998;Shamay et al., 2005)
	alpha-microglobulin	10^{-6} M	Primarily hepatocytes; others kidney	(Tejler et al., 1978;Akerstrom et al., 2000;Larsson et al., 2004;Meining and Skerra, 2012)
	LDL and HDL	10^{-11} to 10^{-12}M	Primarily hepatocytes/ enterocytes	(Camejo et al., 1998;Miller and Shaklai, 1999;Rader, 2006)

Extracellular proteins such as haptoglobin (Hp; that binds to hemoglobin, avoiding heme release) (Okuda et al., 1992;Okazaki et al., 1997;Mollan et al., 2014), hemopexin (Paoli et al., 1999;Shipulina et al., 2000), albumin (Hanson et al., 2011)**,** alpha-1-microglobin (α_1m) (Allhorn et al., 2002;Allhorn et al., 2003) and lipoproteins (Camejo et al., 1998) were also proposed to have such a function (**Table 3**).

4.1 Intracellular Heme Binding Proteins

In mammals, glutathione S-Transferase (GST; EC 2.5.1.18) is expressed in different tissues and cells and belongs to the family of the detoxification enzymes that catalyze phase II conjugation of reduced glutathione (GSH) with a variety of endogenous and exogenous electrophiles, playing a crucial role in the detoxification of xenobiotics (Caccuri et al., 1990;Board and Menon, 2013). This enzyme is also involved in the elimination of toxic by-products generated in oxidative reactions (Caccuri et al., 1990;Board and Menon, 2013), by binding to xenobiotics and porphyrins, including heme (Caccuri et al., 1990). Heme binding to GSTs can occur *via* two different binding sites, one with low affinity ($K_D=10^{-6}$ M) and another with high ($K_D=10^{-8}$M) affinity (Caccuri et al., 1990). Moreover, in the presence of glutathione the high affinity heme-binding site increases even further its affinity towards heme, presumably due to conformational changes upon glutathione binding. This supports the notion that GST is a heme binding protein and that glutathione acts as a modulator of heme binding to GST (Caccuri et al., 1990), regulating heme transport from the mitochondria to other cellular compartments through a still unknown mechanism (Kirschner-Zilber et al., 1989;Boyer and Olsen, 1991).

HBP23 is a stress-induced multifunctional antioxidant peroxiredoxin that binds heme with high affinity ($K_D\approx10^{-8}$M) (Iwahara et al., 1995;Hirotsu et al., 1999). Peroxiredoxins are expressed in a wide variety of tissues, including liver, kidney, spleen and heart (Iwahara et al., 1995;Immenschuh et al., 1997). Heme binding to HBP23 inhibits its antioxidant activity, suggesting an alternative mechanism of heme-induced cell damage, *via* inhibition of cellular antioxidants mechanisms (Ishii

et al., 1995). However, further studies are required to clarify the impact of heme binding to HBP23 on its antioxidant activity.

The p22 heme binding protein (p22HBP) is a cytosolic protein particularly abundant in the liver, but also highly expressed in hematopoietic tissues (*e.g.* fetal liver, bone marrow), retina, pineal gland, erythrocytes and other tissues with presumably high heme metabolism. P22HBP binds to heme with moderate affinity (10^{-6} to 10^{-7} M) (Jacob Blackmon et al., 2002;Dias et al., 2006). Interestingly, this protein seems to have a crucial role in heme biosynthesis during erythroid differentiation. This notion is supported by the demonstration that knock-down of the p22HBP gene in murine erythroleukemia (MEL) cells leads to a decrease in heme biosynthesis and cellular heme levels, while its overexpression during erythroid differentiation leads to elevated heme biosynthesis and hemoglobin production (Taketani et al., 1998). Altogether, these data suggested that p22HBP promotes heme biosynthesis, increasing heme content during erythoid differentiation (Taketani et al., 1998). Moreover, the high levels of p22HBP messenger ribonucleic acid (mRNA) found in the liver suggest a role in hepatic heme metabolism, such as heme binding and/or transport (Taketani et al., 1998). Nevertheless, the precise biological function of p22HBP remains to be clarified. A protein sharing 40% sequence identity to p22HBP, named heme-binding protein 2 (SOUL), has been suggested to bind heme as well (Sato et al., 2004), but this proposal has recently been challenged (Ambrosi et al., 2011).

Fatty acid binding protein (FABP) interacts with and modulates fatty acid metabolism, thus preventing their cytotoxic effects (Listenberger et al., 2003;Martins de Lima et al., 2006). FABP also binds heme, with an affinity that varies according to the oxidative state of Fe (K_D~ 10^{-7} M to Fe^{3+} and 10^{-8} M to Fe^{2+}) (Borchers and Spener, 1993). Once bound to heme, FABP is no longer able to bind fatty acids, suggesting that the same binding site might be shared between heme and fatty acids (Stewart et al., 1996;Thompson et al., 1999). While FABP is known to facilitate fatty acid transport, whether the same applies to heme remains unknown (Stewart et al., 1991).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is a heme binding protein with a heme affinity of around 10^{-8} M (Chakravarti et al., 2010;Hannibal et al., 2012) highly expressed in tissues with high energy requirement (*e.g.* muscle, heart, and brain)(Tristan et al., 2011). This protein is involved in the insertion of heme into the inducible nitric oxide synthase (iNOS; EC 1.14.13.39), promoting iNOS maturation and function (Chakravarti et al., 2010). This process is regulated by the product of iNOS, NO (Albakri and Stuehr, 1996;Waheed et al., 2010), *via* S-nitrosylation of GAPDH and consequent inhibition of heme delivery to iNOS (Chakravarti et al., 2010), as a negative feed-back mechanism. A more recent study has shown that the heat-shock protein 90 (HSP90) presents a similar function, acting in a nucleoside triphosphate (ATP) dependent manner (Ghosh et al., 2011). HSP90 is also needed for heme insertion into soluble guanylyl cyclase (sGC; EC 4.6.1.2), a step which is necessary to support sGC maturation and function (Ghosh and Stuehr, 2012).

Biliverdin reductase (BVR; EC 1.3.1.24) catalyzes the conversion of biliverdin into the antioxidant bilirubin, but also has a wide range of other functions (Kapitulnik and Maines, 2009). More recently, BVR was shown to bind heme with an affinity of 10^{-7} M, transporting heme to the nucleus for the induction of HO-1 protein in a heme-dependent manner (Tudor et al., 2008).

4.2 Extracellular Heme Binding Proteins

Extracellular heme binding proteins are present mainly in the plasma, where they bind heme released by oxidized hemoproteins, such as cell-free hemoglobin produced during hemolysis, ensuring its delivery to cells for recycling into hemoproteins or for degradation by the HO system (**Fig. 7**; **Table 3**).

Haptoglobin is an acute phase glycoprotein that binds hemoglobin dimers, preventing the release of its heme groups during hemolysis (Tolosano et al., 2002;Andersen et al., 2012a). Haptoglobin exists mainly in three different and highly stable haplotypes, namely Hp1-1 (dimers), Hp2-1 (oligomers) and Hp2-2 (oligomers), with similar affinity for hemoglobin ($K_D \approx 10^{-12}$M) (Okuda et al., 1992;Kristiansen et al., 2001;Mollan et al., 2014). The hemoglobin-haptoglobin

complex is recognized by the cluster of differentiation (CD)163 (CD163), a scavenger receptor present at the surface of Mø and other cells such as hepatocytes. CD163 promotes endocytosis of the hemoglobin-haptoglobin complex, followed by lysosomal proteolysis of the globin protein and degradation of heme (**see section 5**) (Thomsen et al., 2013). Upon haptoglobin saturation, excess free hemoglobin undergoes auto-oxidation, leading to heme release and production of reactive oxygen species (ROS) (Ferreira et al., 2008;Silva et al., 2009;Gozzelino et al., 2010).

Hemopexin is an acute phase β-1B-glycoprotein with high affinity for heme (K_D<10^{-12}M) found at a concentration of 0.6 to 1.2 g/L in the plasma, under steady state conditions (Paoli et al., 1999;Shipulina et al., 2000). This protein is perceived as the first line of defense against “free heme” (Tolosano et al., 1999;Vinchi et al., 2008). The hemopexin-heme complex binds to the low-density lipoprotein receptor-related protein (LRP)/CD91 expressed at the surface of Mø, hepatocytes and neurons, resulting in its internalization by endocytosis (Hvidberg et al., 2005). Heme dissociates from hemopexin in lysosomes and is subsequently degraded by HO-1 (Hvidberg et al., 2005).

Albumin is the most abundant heme binding protein in the plasma (about 43 g/L) and has an important role in the maintenance of plasma osmotic pressure as well as in the regulation of fluid distribution between body compartments. Albumin has also a high antioxidant capacity and (pseudo) enzymatic activity conferred by the transient binding to heme (Bourdon et al., 1999;Ascenzi et al., 2013). Additionally, human serum albumin has a high ligand-binding capacity for several endogenous and exogenous compounds such as fatty acids, bilirubin, metal ions, steroid hormones, several vitamins and drugs (Wardell et al., 2002;Fasano et al., 2005). Human serum albumin binds heme with an affinity of 10^{-8} M (Adams and Berman, 1980). Its high concentration might compensate for the lower heme affinity making it a potent candidate for the major physiologic heme-binding protein in the serum acting as an important heme scavenger under conditions where hemopexin is saturated (Chow et al., 2008;Huang et al., 2014). Whether human serum albumin regulates heme delivery to cells remains to be established, even though the

presence of a human serum albumin receptor has been proposed, *i.e.* albumin binding protein and neonatal Fc receptor (FcRn). These can potentially mediate the intracellular import of albumin-heme, thus allowing its recycling (Gekle et al., 1995;Tiruppathi et al., 1996;Chaudhury et al., 2006). The mechanism regulating albumin/FcRn binding is still not fully understood, however some studies have demonstrated that this binding is dependent on the pH and occurs in a non competitive manner to immunoglobulin G (IgG), another protein that binds to FcRn. This interaction would allow albumin-heme endocytosis and recycling (Chaudhury et al., 2006;Andersen et al., 2012b). Alternatively and most probably, albumin transfers heme directly to other heme scavengers such as hemopexin (Noyer et al., 1998).

α1-microglobin is another extracellular heme binding protein with the lowest known affinity for heme (K_D of 10^{-6} M), when compared to other plasma heme scavengers (Larsson et al., 2004;Olsson et al., 2011). This protein has a truncated form (t-α1microglobin) that lacks the C-terminal tetrapeptide (Leu-Ile-Pro-Arg; LIPR) and has a free Cys (C^{34}) that is presumably removed by oxyhemoglobin cleavage. Nonetheless, this truncated form retains not only the heme binding properties of the full-length protein but in addition, acquires heme catabolism capacity (Allhorn et al., 2002). Presumably therefore, the truncated form of α1microglobin is involved in the catabolism of "free heme", providing an extracellular mechanism for heme detoxification, activated directly by the accumulation of oxidized cell-free hemoglobin in plasma (Allhorn et al., 2002).

High (H) and low (L) density lipoproteins (DL) bind heme with high affinity ($K_D \approx 10^{-11}$ to 10^{-12}M)(Camejo et al., 1998), with faster kinetics than any other plasma heme scavenger. This suggests that, as it accumulates in the plasma, "free heme" would primarily bind to lipoproteins and then be transferred to hemopexin or albumin (Camejo et al., 1998). However, heme binding to lipoproteins can induce their oxidation, *via* heme-Fe or Fe release from heme, with the generation of toxic products. Lipoproteins can be highly cytotoxic upon oxidation, leading to cell death and promoting inflammation (Balla et al., 1991;Camejo et al., 1998;Jeney, 2002) a

deleterious effect that is prevented by their uptake by Mø *via* the CD36 receptor (Calvo et al., 1998;Camejo et al., 1998).

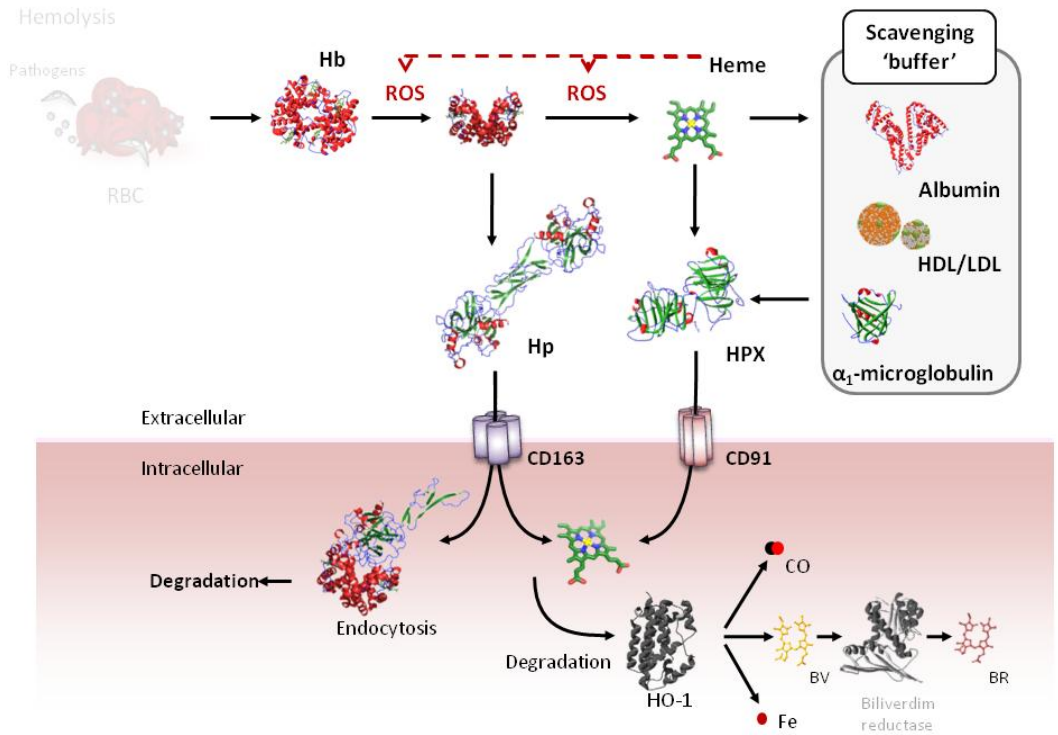


Figure 7: Heme scavengers. There are several extracellular proteins that can bind heme transiently conferring protection against "free heme" released from cell free Hb during hemolysis. Cell free Hb is rapidly dissociated into dimers that can be scavenged by haptoglobin (Hp). The Hb-Hp complex is then uptaken by CD163 pathway for endocytosis and degradation, preventing cell free Hb accumulation in plasma. When Hp is saturated, cell free Hb accumulates in plasma and upon oxidation releases heme, which is uptaken by hemopexin (HPX). The complex HPX-Heme is taken up by CD91 receptor, delivering heme for degradation by HO-1. There are other heme scavengers such as albumin, lipoproteins (LDL, HDL) and α_1-microglobulin that presumably bind heme transiently. Whether heme bound to these scavengers can be directly uptaken by cells or whether this requires heme transfer to HPX remains to be elucidated.

Understanding the dynamics of heme transfer between different plasma heme scavengers is crucial to unravel the role of "free heme" under homeostatic and pathologic conditions. The kinetics of heme transfer between the main plasma

heme scavengers, namely hemopexin, albumin and HDL or LDL suggests that about 80% of the heme released from cell-free hemoglobin is uptaken by the lipoproteins, while the remaining “free heme” binds to the other scavengers. However, under steady state conditions, heme distribution is estimated as 2.4%, 18.6%, 44 % and 35% bound to LDL, HDL, human serum albumin and hemopexin, respectively, with a faster uptake of heme by hemopexin and human serum albumin, due to the high dissociation constants of Heme-HDL and –LDL complexes (**Fig. 8**) (Miller and Shaklai, 1999). This suggests that approximately 20% of heme still remains bound to lipoproteins, which can lead to lipid peroxidation (Miller and Shaklai, 1999).

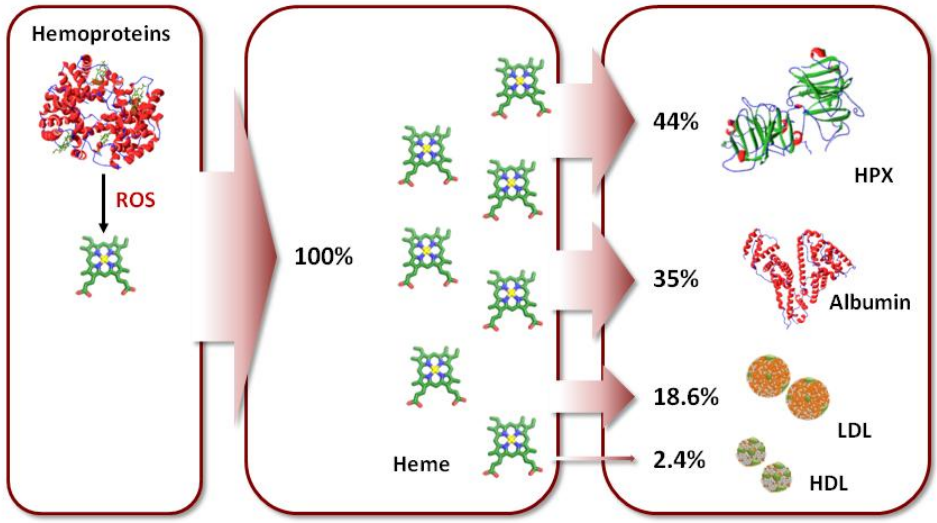


Figure 8: Predicted heme distribution in plasma. When released, 44%, 35%, 18,6% and 2,4% of the “free heme” is bound to hemopexin, albumin, HDL and LDL, respectively. Presumably, heme binds initially to lipoproteins, from which is rapidly uptaken by HPX and albumin. Nonetheless, about 20% of heme remains bound to lipoproteins which might promote lipid peroxidation.

5 *HEME CATABOLISM*

When delivered to cells, heme in hemoglobin-haptoglobin complexes, heme-hemopexin, heme-albumin and/or “free heme” can be degraded *via* the HO system (Otterbein et al., 2003;Seixas et al., 2009;Gozzelino et al., 2010;Fraser et al., 2011) or presumably re-used in the *de novo* synthesis of hemoproteins. Heme can also be degraded by a non-enzymatic mechanism, mediated for instance by H_2O_2 and/or hypochlorous acid (HOCl) (Nagababu and Rifkind, 2000b;a;2004;Maitra et al., 2011) as well as by glutathione (Atamna and Ginsburg, 1995) (**Fig. 9**).

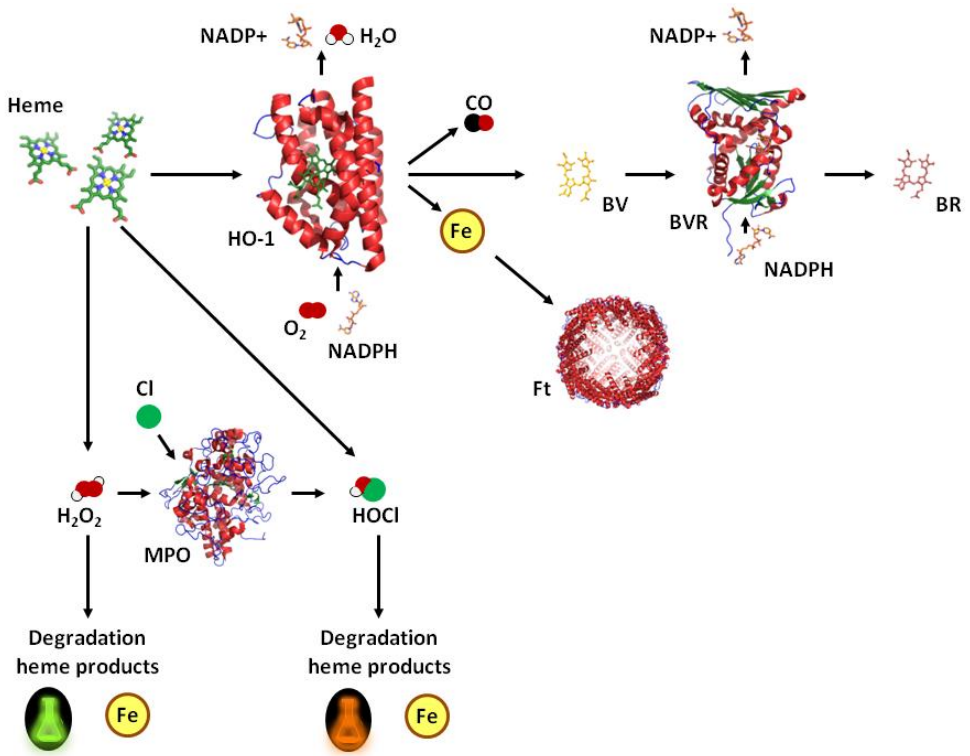


Figure 9: Heme catabolism. The majority of free heme is degraded by heme oxygenases, which catalyze heme catabolism into carbon monoxide (CO), Fe and biliverdin (BV), *via* a reaction assisted by adenine dinucleotide phosphate nicotinamide (NADPH) and oxygen (O_2). The released Fe is storaged in ferritin (Ft) while BV is converted into the antioxidant bilirubin (BR) by biliverdin reductase (BVR), *via* a reaction assisted by NADPH. Heme can also be degraded by hydrogen peroxide (H_2O_2) and/or hypochlorous acid (HOCl) with the concomitant formation of Fe and different fluorescent heme degraded products.

5.1 Enzymatic Mechanism of Heme Catabolism – Heme Oxygenases

Heme Oxygenases (HO, EC 1.14.99.3) are microsomal enzymes that convert heme into CO, Fe and biliverdin, as first described by Tenhunen and co-workers (**Fig. 7 and 9)** (Tenhunen et al., 1968;Yoshida et al., 1974). Three isoforms of HO were identified so far: HO-1 encoded by *HMOX1*, HO-2 encoded by *HMOX2* and HO-3 encoded by *HMOX-3a* and *HMOX-3b* genes. The latter are processed pseudogenes that derive from *HMOX-2* transcripts, comprising exons 2 through 5 (no exon 1) of *HMOX-2* and lacking introns, hence suggesting that there is no functional HO-3 protein (Scapagnini et al., 2002;Hayashi et al., 2004). Further studies are required to understand how the *Hmox-3* pseudogenes were generated. Under homeostasis, most cells express HO-1 at low or undetectable levels, whereas HO-2 is constitutively expressed (Maines et al., 1986). Regarding HO-3, its expression in tissues is still unclear and further studies are required to characterize it.

HO-2 activity seems to be more important to maintain heme levels under steady state conditions, while HO-1 is induced upon oxidative stress, heat shock, hypoxia and increased heme bioavailability (Applegate et al., 1991). Therefore, HO-1 is classified as a stress response protein with an essential role in protecting cells against different forms of stress (Otterbein et al., 2003;Seixas et al., 2009;Soares and Bach, 2009;Gozzelino et al., 2010). The protective effects exerted by HO-1 act *via* different mechanisms that include inhibition of heme accumulation (Otterbein et al., 2003;Seixas et al., 2009;Dunn et al., 2014) and redox activity, by the biliverdin/bilirubin system (McDonagh, 2010) or upon CO binding (Soares et al., 2002;Pamplona et al., 2007). CO has a high affinity for ferrous-heme, and once bound to the Fe in the heme group of hemoproteins, prevents its oxidation, thus inhibiting heme release (Pamplona et al., 2007;Ferreira et al., 2008;Ferreira et al., 2011). This protective effect of CO is crucial to prevent the pathogenesis of several immune-mediated inflammatory diseases associated with hemolysis, as it inhibits heme release from hemoglobin (Pamplona et al., 2007;Ferreira et al., 2008;Ferreira et al., 2011;Jeney et al., 2014).

Heme degradation by HO generates two anti-oxidants, namely, biliverdin and bilirubin, produced *via* reduction of biliverdin in the presence of Nicotinamide adenine dinucleotide phosphate (NADPH) by BVR (Stocker et al., 1987). Bilirubin is a stronger antioxidant than biliverdin, preventing damage associated with lipid and protein oxidations (Neuzil and Stocker, 1994). In several immune-mediated inflammatory diseases, the biliverdin/bilirubin system was shown to have a cytoprotective effect, presumably by preventing oxidative damage (Ollinger et al., 2007;Li et al., 2014b). Nevertheless, this protective mechanism still remains poorly understood.

Another end product of heme degradation, Fe, is a potent pro-oxidant prone to generate harmful hydroxyl radicals through the Fenton reaction (Fenton, 1894) and more precisely through a process called “Haber-Weiss-cycle” (Kehrer, 2000). For that reason, cellular Fe content must be tightly regulated (Hentze et al., 2004), limiting its availability through storage into Fe storage proteins, such as ferritin (Arosio and Levi, 2002) or *via* extracellular Fe transport by ferroportin (Mitchell et al., 2014).

Ferritin is a multimeric protein (24-subunits) composed by heavy/heart chains (FtH) and light/liver chains (FtL), with high capacity for Fe storage (about 4500 Fe ions *per* ferritin complex). The proportion of L-/H-chains depends on cell and tissue type, as well as their Fe content and varies among the different organisms. Ferritin with a higher proportion of FtH has higher ferroxidase activity due to the catalytic properties of FtH that converts Fe^{2+} into Fe^{3+}, while ferritins with higher proportion of FtL promote Fe^{3+} nucleation and storage (Wang et al., 2006). Ferritin expression is coupled to that of HO-1, and while HO-1 is up-regulated by heme, Ft expression is up-regulated by end product of heme catabolism, Fe (**see section 5.1**) (Hintze et al., 2007).

5.2 Non-Enzymatic Mechanism of Heme Degradation

5.2.1 Hydrogen Peroxide (H_2O_2)

H_2O_2 is generated *via* dismutation of superoxide (O_2^-) or catalyzed by the action of superoxide dismutase (SOD, EC 1.15.1.1), amine oxidase (EC 1.4.3.6) or glucose oxidase (EC 1.1.3.4) (Segura-Aguilar, 1993;Rypniewski et al., 1995;Salazar and Van Houten, 1997;Pietrangeli et al., 2000). In erythrocytes, H_2O_2 is produced mainly by the auto-oxidation of oxyhemoglobin (Misra and Fridovich, 1972) which leads to a cascade of reactions resulting in the production of fluorescent products belonging to the degradation of heme moiety, *i.e.* the protoporphyrin IX structure, amongst other product (Nagababu and Rifkind, 1998). Heme reaction with H_2O_2 generates dipyrrolic propentdyopents, hematic acid and methylvinylmaleimide molecules, but further experiments are required to address the correct identity of other end products of this reaction (Nagababu and Rifkind, 2000b;a;2004) (**Fig. 9**).

5.2.2 Hypochlorous (HOCl)

Myeloperoxidase (MPO, EC 1.11.2.2), an enzyme predominantly expressed in neutrophils, catalyzes the conversion of H_2O_2 into HOCl in the presence of the chloride anion. HOCl is a powerful oxidant, which is important in the defense against microbial organisms (Albrich et al., 1981). HOCl has also been implicated in heme degradation by direct interaction with the tetrapyrrole ring of heme in hemoproteins (*e.g.* hemoglobin), leading to Fe release (Pullar et al., 2000). Presumably other molecules are generated from the tetrapyrrole ring destruction (Maitra et al., 2011;Abu-Soud et al., 2014) but their identity remains unknown (**Fig.9**).

6 *HEME AS A PATHOPHYSIOLOGICAL MOLECULE*

Several diseases are associated with disruption of the homeostatic parameters that compromise RBC integrity, causing hemolysis and release of hemoglobin into the plasma (**Fig. 10, 11 and 12**). Considering that in humans, one fourth of the total cells are RBC (2-3x10^{13} in adult) that have exceedingly high levels of hemoglobin (3x10^{8} protein molecules/RBC) and heme (1.2x10^{9} heme/RBC) contents, this results in rather small levels of hemolysis being associated with the generation of high levels of cell-free hemoglobin which presumably leads to "free heme" accumulation (Gozzelino and Soares, 2014).

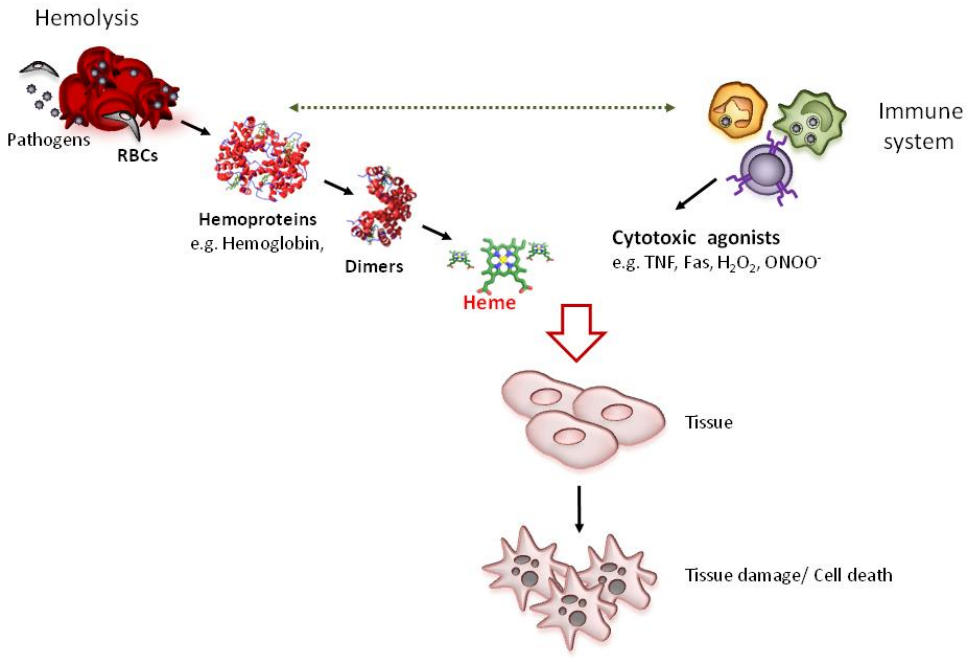


Figure 10: Pathophysiologic effect of heme. Under hemolytic conditions heme is released from oxidized hemoglobin (Hb) dimers and accumulates in plasma, acting in pro-oxidant and pro-inflammatory manner. In presence of other cytotoxic agonists produced during inflammation, heme triggers programmed cell death (Gozzelino and Soares, 2011).

This non-hemoglobin bound heme, hereby referred to as “free heme”, can act in a pro-inflammatory (Graca-Souza et al., 2002;Figueiredo et al., 2007) and cytotoxic manner (Seixas et al., 2009;Gozzelino et al., 2010;Larsen et al., 2010), contributing to the pathogenesis of several immune-mediated inflammatory diseases. These include sickle cell disease (Vinchi et al., 2013), malaria (Pamplona et al., 2007;Ferreira et al., 2011) and severe sepsis (Larsen et al., 2010), which probably comprise only a few among many others. Other sources of “free heme” cannot be excluded, as release upon oxidation of hemoproteins such as myoglobin from muscle cells, which contributes to the pathogenesis of rhabdomyolysis (Adornato et al., 1978;Nath et al., 1992).

6.1 Pro-Oxidant Activity of Heme

The pro-oxidant activity of heme is driven by the participation of its Fe in the Fenton reaction (Fenton, 1894)/“Haber-Weiss” (Kehrer, 2000) reaction (**Fig. 11**). Whether this is driven by heme-Fe itself, or by Fe when released from heme remains to be elucidated. In any case, heme-driven ROS generation can catalyze the oxidation of a variety of macromolecules, compromising their biological function, as demonstrated for proteins (Aft and Mueller, 1984), deoxyribonucleic acid (DNA) (Glei et al., 2006) and lipids (Gutteridge and Smith, 1988;Mashima et al., 2002). The latter are of particular importance, as the hydrophobic properties of heme favor its intercalation into phospholipid bilayers (Balla et al., 1991;Jeney et al., 2002), catalyzing lipid peroxidation and the concomitant generation of toxic compounds, enhancing membrane permeability and disrupting cell membrane components (Schmitt et al., 1993;Kumar and Bandyopadhyay, 2005). This is particularly relevant to RBC where heme can promote hemolysis (Chou and Fitch, 1981). This occurs *via* a peroxidation process targeting RBC membranes as well as the cytoskeleton, which cross-links and aggregates spectrin and protein 4.1 but not actin, thus compromising RBC integrity (Solar et al., 1990). Heme can also target DNA, namely mitochondrial DNA (mtDNA) (Suliman et al., 2002), leading to its deletion and eventually to programmed cell death (Suliman et al., 2002). Apart from RBC, the deleterious effect of “free heme” is also exerted in many other cell types,

including renal epithelial (Tracz et al., 2007), neuron-like (Goldstein et al., 2003) and cardiac cells (Bhoite-Solomon et al., 1993).

Heme accumulation in plasma promotes the oxidation of lipoproteins, such as LDL and HDL (Camejo et al., 1998) and, in the presence of H_2O_2, generates conjugated dienes, thiobarbituric acid reacting substances, and F2-isoprostanes (Camejo et al., 1998). Oxidized LDLs are uptaken by Mø, leading to their conversion into foam cells, which are known to contribute to the development of pathologic conditions such as atherosclerosis (Miller and Shaklai, 1994;Kumar and Bandyopadhyay, 2005). Besides that, heme-driven LDL peroxidation leads as well to the generation of highly cytotoxic products that are deleterious to vascular endothelial cells (Balla et al., 1991;Jeney et al., 2002).

The cytotoxic effects of heme are exacerbated by the concerted action of pro-inflammatory agonists, such as tumor necrosis factor (TNF) and Fas, among others (Larsen et al., 2010;Gozzelino et al., 2012). This synergistic effect sensitizes cells (*e.g.* primary hepatocytes, pancreatic β cells, Human Embryonic Kidney 293 cells and oligodendrocytes) to undergo programmed cell death, presumably *via* a mechanism involving the unfettered production of free radicals (Gozzelino et al., 2010;Larsen et al., 2010). This notion is supported by experiments in which heme sensitization of TNF-mediated programmed cell death is inhibited by the antioxidant N-acetylcysteine (NAC) (Seixas et al., 2009;Larsen et al., 2010;Gozzelino and Soares, 2011). Presumably, the cytotoxic effect of heme relies on sustained ROS-driven activation of c-Jun N-terminal Kinase (JNK) signaling transduction pathway, most likely *via* inhibition of phosphatase enzymes that regulate JNK activation in response to TNF (Kamata et al., 2005;Gozzelino et al., 2012), as demonstrated *in vitro* and *in vivo* in Hepa 1-6 cells and mice, respectively (Gozzelino et al., 2012). Sustained JNK activation promotes the activation of effector caspases, namely caspase 3 (EC 3.4.22.56), that triggers programmed cell death *via* apoptosis (Gozzelino et al., 2012;Gozzelino and Soares, 2014). This cytotoxic effect can be prevented through the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), a transcription factor involved in the inhibition of TNF induced programmed cell death (Kamata et al., 2005;Gozzelino et al., 2012).

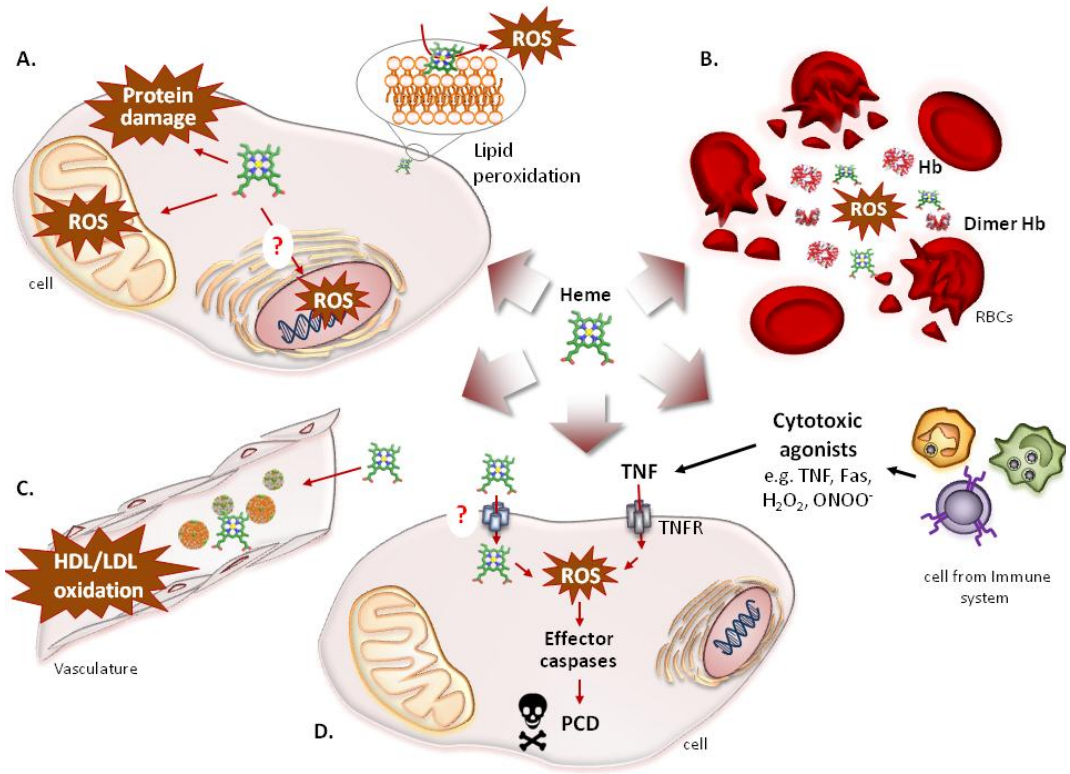


Figure 11: Pro-oxidant effect of heme. "Free heme" is reactive, promoting oxidative stress, *via* a poorly understood mechanism, driven by the heme-Fe. Heme accumulation under hemolytic conditions leads to **A.** cellular damage via the generation of reactive oxygen species (ROS) and leading to DNA and protein damage as well as lipid peroxidation. **B.** Heme is cytotoxic for RBC, exacerbating hemolysis. **C.** Heme promotes the expression of pro-inflammatory genes in vascular endothelial cells as well as oxidation of lipoproteins (*e.g.*LDL), contributing to vasoconstrictions. **D.** Heme also sensitizes parenchyma cells to undergo tumor necrosis factor (TNF) mediated programmed cell death (PCD), *via* ROS formation.

The protective mechanism of NF-κB is mediated by the expression of anti-oxidant genes, such as manganese superoxide dismutase, FtH (Gozzelino and Soares, 2014) and X-chromosome-linked inhibitor of apoptosis (XIAP) (Tang et al., 2001), among others. Recently, it has been demonstrated that Mø undergoes necroptosis when exposed to heme, through two independent but complementary mechanisms,

one involving Toll-like receptor 4 (TLR-4) signaling, leading to the production of TNF and another by ROS production induced by heme (Fortes et al., 2012). Furthermore, this cytotoxic effect is dependent on TNF mediated cell death by receptor-interacting protein (RIP)1 and RIP3 proteins as well as JNK activation (Fortes et al., 2012).

6.2 Pro-Inflammatory Properties of Heme

Heme can also act in a pro-inflammatory manner, promoting the activation of Mø and neutrophils as well as endothelial cells and possibly other cell types (**Fig. 12**) (Wagener et al., 2001;Graca-Souza et al., 2002;Belcher et al., 2014;Chen et al., 2014;Dutra and Bozza, 2014). In endothelial cells, heme can activate the expression of P-selectin and von Willebrand factor (VWF) release from Weibel-Palade bodies, *i.e.* endothelial cell storage granules, *via* a mechanism dependent of TLR4/Myd88 pathway (Belcher et al., 2014). Heme driven degranulation of Weibel-Palade bodies is mediated *via* a mechanism that involves the activation of protein kinase C (PKC, EC 2.7.11.13), NADPH oxidase (NOX, EC 1.6.3.1) and ROS production (Belcher et al., 2014). Furthermore, heme promotes the expression of adhesion molecules, such as vascular cell adhesion molecule 1 (ICAM-1) and intercellular adhesion molecule 1 (ICAM-1) as well as leukocyte rolling and adhesion (Belcher et al., 2014). This mediates neutrophil adhesion to endothelium cell and its emigration from blood vessels.

Heme is also able to active neutrophils *via* a mechanism involving the induction of PKC signal transduction pathway with ROS generation and actin polymerization as well as chemotaxis stimulation (Graca-Souza et al., 2002). Recent studies have shown that heme promotes neutrophil chemotaxis through the production of pro-inflammatory mediator leukotriene B4 (LTB4) (Monteiro et al., 2011) or acting as a chemoattractant (Porto et al., 2007). Heme also stimulates the induction of superoxide anion production by neutrophils through an NOX dependent mechanism, leading to an oxidative burst (Graca-Souza et al., 2002). Additionally, heme can induce the production of chemokines, such as interleukine 8 (IL-8) by a process independent of PKC activation, promoting neutrophil chemotaxis and

priming neutrophils to undergo oxidative burst as well as adhesion to endothelial cells (Graca-Souza et al., 2002). IL-8 is also expressed in endothelial cells upon heme stimulation, presumably *via* the induction of oxidant-sensitive mechanisms, and inhibition of the transcription factor hypoxia inducible factor-1 (HIF-1) (Natarajan et al., 2007). This chemokine was shown to promote angiogenesis, encompassing endothelial cell proliferation, migration and increasing membrane permeability (Li et al., 2003). More recently, heme was shown to induce the formation of neutrophil extracellular traps (NETs), presumably *via* ROS production in neutrophils (Chen et al., 2014). NETs are extracellular fibers composed of decondensed chromatin and granular enzymes that confer host resistance to bacterial infection (Brinkmann et al., 2004). However, NETs can also be harmful to the organism, promoting inflammation and eventually trapping of self compounds, such as RBC (Fuchs et al., 2013). This was correlated with the pathogenesis of several non-infectious diseases, where the pathologic effect associated with NETs formation might be triggered by heme accumulation in plasma, as suggested for sickle cell disease (Chen et al., 2014). Yet another pro-inflammatory effect of heme relies on its ability to prolong neutrophil survival (Arruda et al., 2004), presumably *via* modulation of signaling pathways involved in cell survival, such as those involving the major mitogen-activated protein kinases (MAPKs), namely extracellular signal-regulated kinase (ERK)) and protein kinase B (Akt, EC 2.7.11-12)/Phosphoinositide 3-kinase (PI3K, EC 2.7.1.137) pathways as well as the activation of NF-κB (Arruda et al., 2004;Arruda et al., 2006). This effect seems also to require the maintenance of mitochondrial integrity (Arruda et al., 2004;Arruda et al., 2006). In summary, accumulation of heme in plasma or tissues can lead to an uncontrolled activation of neutrophils and deleterious inflammation, compromising homeostasis through a variety of different mechanisms.

The pro-inflammatory effect of heme was also shown to involve TLR4 signaling, through activation of myeloid differentiation 88 (MyD88) pathway, involving MAPK and NF-κB and leading to the induction of pro-inflammatory gene expression including TNF and keratinocyte derived chemokine (KC) (Figueiredo et al., 2007;Belcher et al., 2014). TLR recognize pathogen-associated molecular

patterns (PAMPs), *i.e* bacterial lipopolysaccharide (LPS) and intracellular proteins such as high mobility group protein 1 (HMGB1), biglycan, oxidized LDL, fibrogen and monosodium urate crystals (Piccinini and Midwood, 2010). TNF production through TLR4 mediated heme activation is dependent of its Fe and the vinyl group (Figueiredo et al., 2007). Whether this occurs *via* direct interaction remains to be elucidated. Heme also synergizes with several cell surface (TLR2, TLR4, TLR5), endosome (TLR3, TLR9) and cytosolic (NOD1, NOD2) receptors to trigger the secretion TNF and IL-6 as well as IP-10 cytokines by MyD88 and TIR (Toll–IL-1 receptor)-domain-containing adapter-inducing interferon (TRIF)- dependent mechanism (Fernandez et al., 2010).

IL-1β is another cytokine induced by heme in LPS primed Mø, *via* a mechanism involving the activation of another pattern recognition receptor (PRR), namely the nucleotide binding domain and leucine rich repeat containing family pyrin domain containing 3 (NLRP3) inflammasome (Dutra et al., 2014). This effect was also shown to be dependent on mitochondrial ROS production, potassium efflux, NADPH oxidase-2 and activation of spleen tyrosine Kinase (Syk, EC 2.7.10.2). Contrarily to a previous work (Li et al., 2014a), heme activation of NLRP3 was unrelated with ATP release, lysossomal damage and purinergic receptor P2X7 activation. These distinct results were correlated the experimental conditions used, namely with the presence/absence of serum in Mø cultures (Dutra et al., 2014;Li et al., 2014a). This suggests that heme activation of NLRP3 can occur by two different mechanisms that are dependent of serum. Alternatively, the results obtained by Li et al. (Li et al., 2014a) can be due to the observation that absence of serum leads to an increase in macrophage cell death with release of ATP and consequently activation of NLPR3 through the receptor P2X7, as suggested by (Dutra et al., 2014).

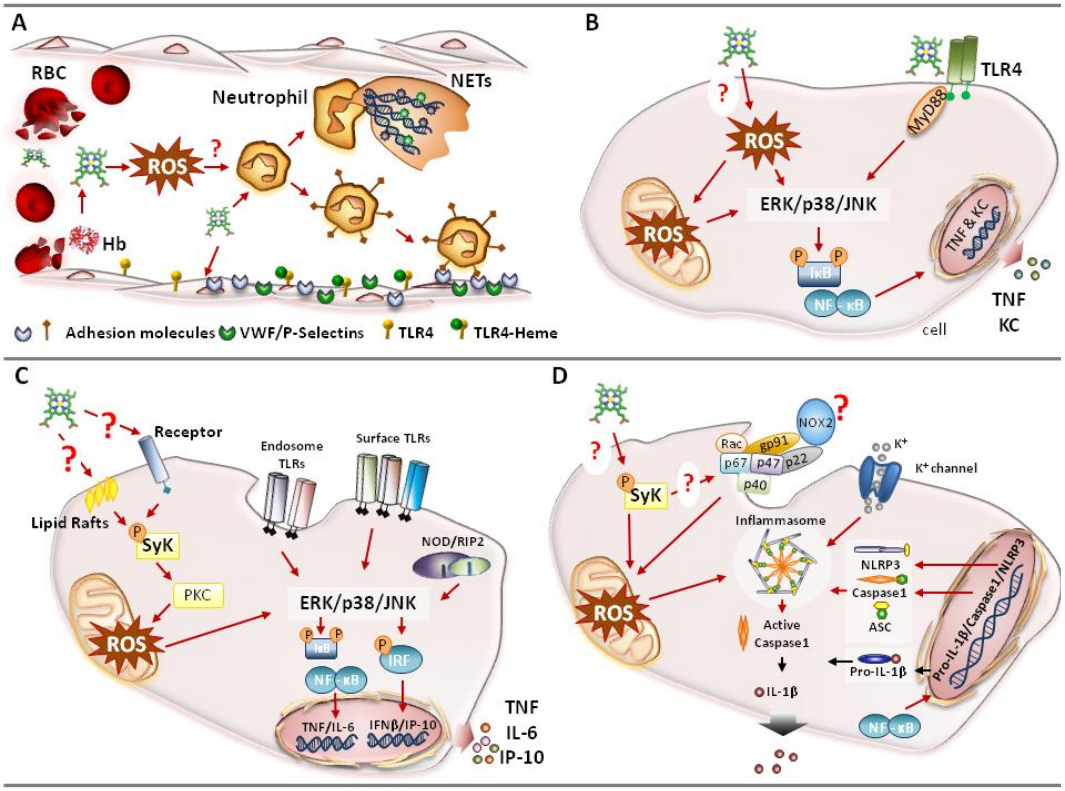


Figure 12: Pro-inflammatory effect of heme. Accumulation of "free heme" in plasma acts in a pro-inflammatory manner *via* **A.** activation of neutrophils and endothelial cells, where it is sensed *via* Toll-like receptor (TLR)4 leading to degranulation of Weibel Palade bodies with the release of P-selectins and Von Willebrand factor (VWF). **B.** Heme sensed by TLR4 in Mø, induces the production of pro-inflammatory cytokines, *i.e.* keratinocyte-derived chemokine (KC) and tumor necrosis factor (TNF), *via* a mechanism involving MyD88 and Mitogen-activated protein kinases (MAPKs; ERK, p38 and JNK) that activate NF-κB. **C.** Heme also promotes the phosphorilation of spleen tyrosine kinase (SyK) by an unknown mechanism. SyK activation triggers the activation of the protein kinase (PKC), inducing ROS production. Amplification of the immune response against microbial molecules is synergized by heme with cell surface (TLR2, TLR4, TLR5), endosome (TLR3, TLR9) and cytosolic (NOD1, NOD2) receptors, increasing the cytokine production (TNF, IL-6, IFNβ, IP-10). **D.** Heme activates the NLRP3 inflammasome with induction of IL-1β *via* a mechanism that involves Syk as well as activation of NADPH Oxidase (NOX) with mitochondrial ROS production. This requires the NF-κB driven NLRP3 expression and activation of caspase-1 *via* a mechanism dependent of K^+ efflux, NOX2 and mtROS as well as Syk activation. (Adapted from (Dutra and Bozza, 2014)).

Another important feature of heme is its ability to activate LPS stimulated B cells, promoting B cell differentiation and class switch recombination, through a mechanism involving the inactivation of the basic leucine zipper transcription factor 2 (Bach2)(Watanabe-Matsui et al., 2011). Bach2 is a transcriptional repressor of B lymphocyte-induced maturation protein-1 (Blimp-1), expressed in the progenitors and mature B-cells, and containing at least four heme-binding domains. Heme binding inactivates Bach2, promoting its nuclear export and degradation through the ubiquitin/proteasome system (Hira et al., 2007;Zenke-Kawasaki et al., 2007). Thereby, expression of Blimp-1 increases, presumably inducing B-cell maturation, while acting as a repressor of antibody class switch recombination. Moreover, Bach2 is required for Ig somatic hypermutation, suggesting that Bach2 inactivation by heme affects antigen-specific antibody production. This suggests that heme is involved in the modulation of activated B cells responses, an effect that might be relevant on the pathogenesis of diseases (Watanabe-Matsui et al., 2011). Once more, further studies are required to address these questions.

7 HEME IN THE PATHOGENESIS OF IMMUNE MEDIATED INFLAMMATORY DISEASES

Several immune mediated inflammatory diseases are associated with hemolysis and subsequently with the release of heme from cell-free hemoglobin (Gozzelino et al., 2010;Larsen et al., 2010). Presumably, heme contributes to the pathogenesis of these diseases, as illustrated for malaria caused by *Plasmodium* infection, severe sepsis caused by polymicrobial infections and sickle cell disease cause by mutations in the β chain of hemoglobin. Several methods were developed for total heme quantification, *i.e.* heme bound or not to molecules such as proteins or lipids, including formic acid assay (Kuross et al., 1988;Regan et al., 2001), pyridine hemochrome assay (Berry and Trumpower, 1987) and fluorescent assay (Vinchi et al., 2013). However, no further methodology was developed to determine and quantify unequivocally heme in its free state, *i.e.* not bound to other molecules, which limits our understanding of the pathologic rule of this molecule in diseases associated with hemolysis.

7.1 Heme and Malaria

Malaria is an infectious disease caused by *Plasmodium* parasites that affects around 300–400 million people per year with an associated 1–3 millions of deaths per year, mainly in children under 5 years old (Murray et al., 2014). *Plasmodium falciparum* is responsible for most of the severe forms of disease cases and deaths associated with malaria (Miller et al., 2002). *Plasmodium* transmission to the human host starts by subcutaneous injection of the parasite by an infected *Anopheles* mosquito. The parasite migrates to the liver and infects hepatocytes, to initiate the liver stage of infection that occurs without discernible clinical symptoms. Multiplication of the parasite inside hepatocytes produces and releases merozoites that infect RBC, initiating the blood stage of infection (Miller et al., 2002). Multiplication of *Plasmodium* in RBC leads to hemolysis and as such, to the accumulation of cell-free hemoglobin in plasma and production of non-hemoglobin bound heme (Ferreira et al., 2008;Seixas et al., 2009). This so-called "blood stage" of infection is associated with the appearance of all the clinical manifestations of malaria (Idro et al., 2005). Several studies in mice argue that accumulation of non-hemoglobin bound heme in plasma contributes critically to the pathogenesis of severe forms of malaria (Pamplona et al., 2007;Ferreira et al., 2008;Seixas et al., 2009;Ferreira et al., 2011). This pathologic effect is countered by the induction of HO-1 and the production of CO, which can suppress the pathogenesis of severe forms of malaria, suggesting that this protective effect is achieved *via* a mechanism that inhibits the pro-inflammatory and cytotoxic effects of "free heme" (Seixas et al., 2009;Gozzelino et al., 2010;Gozzelino and Soares, 2011). Nevertheless, the mechanisms by which heme promotes the pathogenesis of severe forms of malaria are still not fully understood. Even though it has been proposed that it relies mainly on the pro-oxidant effect of heme, whether this effect is triggered be heme or by Fe release from the heme structure by an enzymatic (by HO-1) or non-enzymatic mechanism (Nagababu and Rifkind, 2000a;2004) remains to be elucidated (**see section 4**). Fe, similarly to heme, exerts deleterious effects countered by ferritin, which can explain the finding that FtH confers protection against the development of severe forms of malaria (Gozzelino et al., 2012).

Individuals carrying hemizygous sickle cell mutations (**see section 7.3**) have survival advantage against malaria, presumably *via* a mechanism involving the induction of HO-1 by the accumulation of non-pathological concentrations of heme (**see section 8.2**) released from cell-free hemoglobin (Pamplona et al., 2007;Ferreira et al., 2011). HO-1 provides protection against the pathologic effect of heme, not only by favoring its degradation but also by generating CO, limiting heme accumulation in plasma (**for more details see section 7.3**).

7.2 Heme and Sepsis

Sepsis is a clinical syndrome defined as systemic inflammatory response syndrome (SIRS) triggered by a systemic inflammatory response to a suspected or proven infection (Angus and van der Poll, 2013). Sepsis can progresses to severe sepsis that emerges due to the development of acute organ dysfunction or to septic shock, when also associated to refractory hypotension (Angus and van der Poll, 2013). Sepsis mortality ranges from approximately 24% to 52% (Silva et al., 2004) and is associated with approximately $20x10^6$ of deaths per year worldwide, accounting for one of the most frequent causes of death in hospitalized patients (Angus and van der Poll, 2013). It is currently believed that the pathogenesis of sepsis is predominantly driven by an unfettered immune response to infection that acts in a deleterious, *i.e.* pathological, manner in the host parenchyma, irrespectively of its effect on the pathogen (Larsen et al., 2010;Medzhitov, 2013). Considering the high rate of mortality and despite clinical treatment (Silva et al., 2004), the long term consequences in sepsis survivors (Wang et al., 2014) together with the emergence of a post-antibiotic era with decreasing availability of anti-microbial drugs (Angus and van der Poll, 2013), it is crucial to understand the complex physiological and molecular mechanisms that promote the pathogenesis of this disease.

Similarly to malaria, sepsis is associated with more or less overt hemolysis (Loran et al., 2006), such as driven by the production of pore-forming bacterial cytolysins that damage RBC membranes (Bhakdi et al., 1996), *e.g. Escherichia coli* hemolysin (Bhakdi et al., 1996). This leads to the accumulation of extracellular hemoglobin in plasma, similarly to malaria, which upon oxidation releases its

prosthetic heme groups. Another process that might lead to hemolysis in the context of sepsis is RBC rheology that is promoted by RBC aggregation and alteration in its deformability and shape, mainly caused by ROS and NO production, impairment of calcium homeostasis and alterations in membrane components (Serroukh et al., 2012).

The contributions of the “free heme” to the pathogenesis of sepsis were demonstrated using an experimental mouse model, where sepsis is induced through a cecal ligation and puncture (CLP) (Larsen et al., 2010). Disease severity was associated with RBC rheology and accumulation of hemoglobin as well as heme in plasma. Expression of HO-1 provided tolerance against polymicrobial infection, presumably by preventing the pathologic effect driven by heme (**see section 5**), as demonstrated using *Hmox1* knockout mice. Moreover, administration of the heme scavenger hemopexin to mice subject to CLP also provided protection against sepsis, further supporting the role of heme in the pathogenesis of this disease (Larsen et al., 2010). Interestingly, patients with sepsis were also shown to develop Rhadomyolysis, suggesting myoglobin as an alternative source of heme other then cell-free hemoglobin (Kumar et al., 2009).

The view that, in a similar manner to mice, heme contributes to the pathogenesis of human sepsis is supported by the observation that low levels of hemopexin in plasma at the onset of sepsis are correlated with increased organ dysfunction and mortality (Larsen et al., 2010;Janz et al., 2013). Accumulation of extracellular hemoglobin, such as associated with low levels of expression of the hemoglobin scavenger haptoglobin, also correlates to higher sepsis mortality (Janz et al., 2013). This suggests that hemolysis, cell-free hemoglobin, decrease of hemoglobin and heme scavengers as well as a possible increase on “free-heme” exacerbate oxidative stress and inflammation (**see section 6**), promoting disease progression and ultimately death. In this study the generation of “free heme” and its contribution for the disease is still controversial as the methodology used for its quantification in plasma samples of the individuals has some limitations, which compromises the precision of the measurements (Larsen et al., 2010).

7.3 Heme and Sickle Cell Disease

Sickle cell disease is a common and inherited genetic disease associated with conformational alterations imposed to RBC, resulting from mutations in the genes encoding for the globin chains of hemoglobin. There are several sickle point mutations in the β-chain of hemoglobin including HbS (β6Glu>Val)(Jallow et al., 2009), hemoglobin C (HbC) (β6Glu>Lys) (Verra et al., 2007) and hemoglobin E (HbE) (β26Glu>Lys)(Hutagalung et al., 1999). Sickle cell disease is estimated to affect about 7% of the world population, and every year approximately half a million of newborns carry homozygous single or composite mutations leading to severe forms of the disease (Weatherall and Clegg, 2001). Hemizygous sickle mutations are non-pathologic *per se*, while conferring a strong protection against malaria, explaining why these mutations have been so stringently selected through evolution of human populations in endemic areas of malaria (Arif, 2007).

Mortality associated with the occurrence of sickle cell disease, as driven by homozygous sickle mutation or by composite hemizygous mutations, is age dependent, with an estimated frequency of 90 % at 20 years of age and dropping to 50% after fifty years of age (Platt et al., 1994). The clinical symptoms of sickle cell disease are associated with low-oxygen pressure promoting hemoglobin polymerization, RBC deformation and reduced RBC half-life that eventually lead to hemolysis (Uzunova et al., 2010). Clinical symptoms include susceptibility to severe infections, severe pain during so-called "sickle-cell crisis", high risk of stroke, renal failure and chronic episodes of hemolysis, all associated with acute chest syndrome and high mortality risk.

Presumably, cell-free hemoglobin accumulates in plasma during hemolysis and consequently, “free heme” is generated *via* its oxidation (Hebbel et al., 1988;Reiter et al., 2002). This is associated with Fe overload of tissue (Hebbel et al., 1988;Reiter et al., 2002;Nagababu et al., 2008) and inflammation, promoting the pathogenesis of the disease. Heme can act in a pro-inflammatory manner in the vascular endothelium eventually promoting transient vaso-occlusion episodes, associated with painful crises and other sickle cell disease symptoms (Wagener et al., 2001). Vaso-occlusion is also induced by heme *via* a TLR4/Myd88 driven

mechanism, leading to P-selectin and VWF release as well as by the expression of adhesion molecules (**Fig. 12**) (Belcher et al., 2014). Heme driven TLR4 signaling is also correlated with the development of acute chest syndrome, a hallmark of sickle cell disease (Ghosh et al., 2013;Belcher et al., 2014).

A recent study suggests that heme induced NETs formation is also involved in the pathogenesis of sickle cell disease (Chen et al., 2014). NETs are formed in the lungs as well as in the plasma of sickle cell disease mice, contributing to hypothermia and later to the incidence of lethality. This notion is supported by the observation that sickle cell disease patients accumulate NETs in plasma during acute chest syndrome and other painful crisis, thus supporting the pathologic effect of heme-driven NETs in the clinical outcome of sickle cell disease (Schimmel et al., 2013).

Other pathologic mechanisms that contribute to the outcome of sickle cell disease, include NO scavenging by cell free-hemoglobin, followed by a decrease in NO concentration, which leads to vasoconstriction (Reiter et al., 2002). Besides that, *in vivo* the binding of NO to the heme group of hemoglobin presumably promotes heme oxidation and hence its release from cell-free hemoglobin (Reiter et al., 2002). Moreover, in the vascular endothelium, oxidized hemoglobin might have *per se* a pro-inflammatory effect (Silva et al., 2009), presumably contributing to the pathogenesis of sickle cell disease.

The pathologic effect of heme in the context of this disease can be countered by an adaptive response associated with the induction of the heme catabolizing enzyme, HO-1, and the production of heme catabolism end-products CO and the antioxidant bilirubin, all of which have been shown to suppress the pathologic outcome of sickle cell disease (Soares et al., 2004;Seldon et al., 2007). The finding that this salutary effect is exerted *via* a mechanism that targets heme is supported by the observation that hemopexin administration prevents vascular dysfunction associated with hemolytic diseases (Vinchi et al., 2013). Presumably, heme release from cell-free hemoglobin is also involved in a crucial way in the pathogenesis of other blood disorders besides sickle cell disease, such as β-thalassemia (Nagababu et al., 2008;Vinchi et al., 2013).

It is well established that when present in hemizygous form sickle hemoglobin mutations (sickle cell trait) are protective against malaria. This presumably occurs *via* a mechanism involving the induction of HO-1 by non-toxic levels of heme that accumulate chronically in plasma (**see section 8.2**) (Ferreira et al., 2011). Upon infection, *Plasmodium* triggers hemolysis and the accumulation of heme in plasma, which induces HO-1, promoting heme degradation. However in sickle cell trait individuals, HO-1 expression is induced before infection, therefore preventing heme accumulation in plasma. This presumably protects sickle cell trait individuals from the pathologic effects of heme and progression of the disease to severe forms of malaria (Pamplona et al., 2007;Ferreira et al., 2011).

8 TARGETING HEME IN THE TREATMENT OF IMMUNE MEDIATED INFLAMMATORY DISEASES

Given the contribution of heme to the pathogenesis of several immune mediated inflammatory diseases, therapeutic approaches targeting heme have been investigated with the purpose of limiting the pathologic outcome of these diseases. Such approaches include the administration of compounds that inhibit heme release from hemoproteins (e.g. CO, NO) (Pamplona et al., 2007;Cabrales et al., 2011;Ferreira et al., 2011), induction of HO-1 (Abraham, 2003;Soares and Bach, 2009), heme scavenging (Grinberg et al., 1999), heme transport (Jonker et al., 2002;Doty et al., 2010) or directly targeting heme by using antibodies generated against heme (Leavy, 2010). These different approaches will be discussed below (**Fig. 13**). Nevertheless, it is important to mention that other approaches can be considered such as the inhibition of TLR4 using specific inhibitor, allowing the inhibition of the pro-inflammatory effect of heme mediated by TLR4 (Figueiredo et al., 2007;Dutra and Bozza, 2014). Alternatively, the combination of different approaches should also be considered as such might increase the efficiency of the treatments against the deleterious effect of heme.

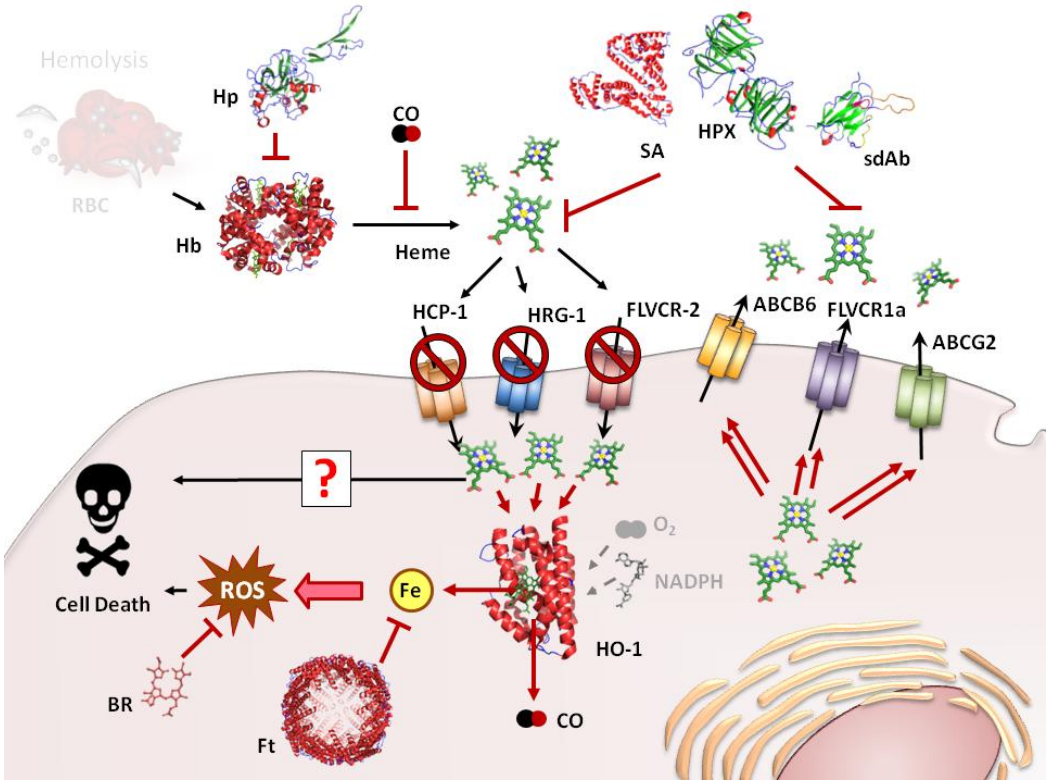


Figure 13: Targeting "free heme". Several mechanisms might be used therapeutically to target directly or indirectly extracellular or intracellular "free heme". The Hb scavenger, Hp prevents the release of heme from Hb while preventing Hb accumulation in the plasma. CO binds the heme groups of Hb with high affinity and prevents its release from Hb. Extracellular heme can also be targeted by two other plasma proteins, *i.e.* albumin and HPX. Inhibition of intracellular transporters, *e.g.* HCP-1, HRG-1 and FLCVR-2 and induction of extracellular heme transporters, *e.g.* ABCB6, FLCVR-1a and ABCG2, might also afford protection against the deleterious effect of free heme. Induction of the heme degradation HO-1 coupled with FT prevents the deleterious effects of heme and Fe, respectively, while generation of bilirubin from heme degradation acts as a potent anti-oxidant. Alternatively, Abs can be used to target heme, inhibiting its deleterious effect.

8.1 Pharmacologic Administration of Gasotransmitters

Several studies support the therapeutic action of CO (Pamplona et al., 2007;Ferreira et al., 2008;Ferreira et al., 2011) and NO (Cabrales et al., 2011) in hemolytic diseases, such as malaria, sickle cell disease and sepsis (Jeney et al., 2014). The salutary effects of CO rely on its cytoprotective effect (Brouard et al.,

2002), its ability to modulate pro-inflammatory responses associated with Mø activation (Silva et al., 2006) and to modulate cell proliferation (Song et al., 2002;Song et al., 2004). In addition, CO can bind to the heme groups of hemoproteins (Queiroga et al., 2012) and suppress their oxidation (Pamplona et al., 2007;Pena et al., 2012;Queiroga et al., 2012). Therefore, when bound to Fe-heme, CO prevents heme release from those hemoproteins, suppressing the pathologic effects associated with this process (Pamplona et al., 2007;Jeney et al., 2014). This protective mechanism should not be limited to hemoglobin, presumably targeting other hemoproteins, such as myoglobin (Kumar et al., 2009). This effect of CO is highly protective against the development of experimental cerebral malaria in mice, suggesting that a similar protective effect maybe operational in humans (Pamplona et al., 2007;Pamplona et al., 2009;Pena et al., 2012). CO exerts salutary effects in other pathologic conditions such as sepsis or sickle cell disease and possibly in other hemolytic disorders associated with more or less severe hemolysis (**see section 7**) (Pamplona et al., 2007;Ferreira et al., 2011). There are however, some limitations to the therapeutic use of CO in that binding of CO to a number of hemoproteins can be cytotoxic (Queiroga et al., 2012). For instance, the high binding affinity of CO to hemoglobin renders it almost irreversible, compromising the ability of hemoglobin to function and carry O_2 to tissues, eventually leading to anoxia and death. Similarly, the binding of CO to heme in myoglobin also compromises its physiologic function, inhibiting not only O_2 storage but also its delivery to muscle cells when high levels of energy are required, such as during exercise.

There is strong evidence for an overall protective action of CO when applied exogenously at sub toxic levels in the context of several immune mediated inflammatory diseases. As such possible therapeutic applications of CO have been explored, even though they still carry numerous limitations (Motterlini and Otterbein, 2010). One of the possible therapeutic applications of CO involves gas inhalation at non-lethal levels (Motterlini and Otterbein, 2010). Alternatively, CO can be administrated using CO-releasing molecules (CORMs) allowing for controlled CO

delivery into cells and tissues (Motterlini and Otterbein, 2010;Garcia-Gallego and Bernardes, 2014).

NO produced by nitric oxide synthases (NOS) can also be applied therapeutically. NO is known to act as a signal transduction molecule involved in diverse physiological processes, such as leukocyte recruitment and migration, smooth muscle and endothelial cell proliferation and migration as well as platelet aggregation. Moreover, NO modulates vascular function through the induction of cGMP production by guanylyl cyclase, which promotes smooth muscle relaxation. On the other hand, NO induces the expression of HO-1 *via* the activation of transcription factor Nuclear factor (erythroid-derived 2)-like 2 (NRF2) (**see section 8.2**) (Jeney et al., 2014). Pharmacologic administration of NO provides protection against severe forms of malaria *via* the induction of HO-1 and the production of CO (Jeney et al., 2014). While the protective effect of NO against cerebral malaria suggests that this molecule can be used therapeutically (Serghides et al., 2011), further studies are required to elucidate the therapeutic benefit of this approach.

8.2 Induction of HO-1

Another therapeutic alternative against the pathologic effect of heme is the induction of HO-1 (Abraham, 2003;Takahashi et al., 2004;Soares and Bach, 2009). This notion is supported by the recurrent observation that HO-1 confers protection against a broad range of pathologic conditions, including malaria, severe sepsis and sickle cell disease (**see section 6**). Induction of HO-1 can be achieved through the administration of non-cytotoxic levels of heme, inhibiting the transcription repressor BACH1 and promoting HO-1 transcription/expression while generating the end-products of heme degradation, CO and bilirubin (**see section 5.2**) (Alam et al., 2003). Alternatively, hemoglobin can be used therapeutically to induce HO-1 (Balla et al., 1995) and prevent the pathogenesis of ischemia/reperfusion injury (Zhou et al., 2002), endotoxic shock (Otterbein et al., 1995) or kidney failure associated with rhabdomyolysis (Nath et al., 1992). This protective effect is mediated by heme release from oxidized hemoglobin (Balla et al., 1995) a

mechanism that underlies the protective effect of sickle cell trait against malaria (**see section 7.3***)* (Ferreira et al., 2011).

HO-1 gene therapy, *via* the expression of HO-1 cDNA using recombinant adenovirus is protective against subarachnoid hemorrhage (Abraham, 2003), Ischemia/reperfusion injury (Coito et al., 2002), kidney transplantation (Blydt-Hansen et al., 2003), hyperoxia-induced lung injury (Gaine et al., 1999) or occlusive vascular lesions (Liu et al., 2002), as demonstrated in rodent models of these diseases. Whether this protective effect is related to heme degradation remains to be elucidated.

Several therapeutic agents induce HO-1, including statins, rapamycin, paclitaxel, NO, aspirin and probucol, among others. For instance, statins induce HO-1 through the modulation of stress responses controlled by p38 MAPK and AKT and involved in the control of cell survival (Lee et al., 2004). This is directly correlated with the inhibition of inflammation and cell proliferation, an effect that should contribute to the ability of statins to provide protection against coronary heart disease. Other drugs have also been shown to regulate HO-1 expression, such as isoflurane, which prevents ischemia/reperfusion injury (Schmidt et al., 2007); paclitaxel, which inhibits atherosclerosis development (Choi et al., 2004) and pyrrolidine dithiocarbamate, which provides ischemic tolerance in the liver (Hata et al., 2003). These drugs might also be considered for the treatment of diseases in which “free heme” acts in a pathologic manner, such as malaria, sepsis and sickle cell disease.

Even though HO-1 induction may prove to be a valuable therapeutic approach there are some limitations that should be considered. For example, polymorphisms in the *HMOX1* gene modulating HO-1 expression as well the response to pharmacological HO-1 inducers are highly prevalent in humans (Exner et al., 2004). Additionally, the physiological consequences of HO-1 over-expression and activity are not fully elucidated, raising questions related to the safety of such therapy. Finally, sustained HO-1 over-expression can promote oxidative stress and cytotoxicity *via* overt Fe production (Suttner and Dennery, 1999).

8.3 Hemoglobin and Heme Scavengers

In pathological condition associated with hemolysis, cell-free hemoglobin accumulation can exhaust the scavenging capacity of haptoglobin. Excess cell-free hemoglobin accumulates in the plasma and eventually undergoes oxidization, leading to the release of heme. This process is inhibited by haptoglobin as suggested by its protective effect against severe sepsis (Janz et al., 2013). Association between cell-free hemoglobin accumulation and higher mortality in sepsis suggests that hemoglobin might act in a deleterious manner, promoting the pathogenesis of this disease, while higher levels of haptoglobin in plasma are correlated with a low mortality rate, presumably related to the ability of haptoglobin to scavenge cell-free hemoglobin (Janz et al., 2013). Moreover, haptoglobin administration is protective against lung injury and inflammation in an experimental model of sepsis in mice (Yang et al., 2003).

When released from extracellular hemoglobin “free heme” can be cleared by specific scavengers such as hemopexin or albumin (Grinberg et al., 1999). Several studies suggest that pharmacologic administration of these heme scavengers improves the outcome of pathologic conditions associated with hemolysis. For example, when used pharmacologically, albumin ameliorates the clinical outcome of severe sepsis (Wiedermann and Joannidis, 2014) as well as that of severe forms of malaria (Maitland et al., 2005). The mechanism *via* which albumin provides protection against the pathogenesis of these diseases remains to be established but this may as well involve the inhibition of the cytotoxic effects of heme.

Alternatively, hemopexin administration might be used to prevent the pathologic effects of “free heme”, as demonstrated for severe sepsis in mice (Larsen et al., 2010). Low levels of hemopexin in patients with severe sepsis, are also associated with increased disease severity (Larsen et al., 2010). Whether hemopexin administration will suppress the pathogenesis of sepsis in humans remains to be established. The beneficial effect of hemopexin might be expanded to other hemolysis-associated pathologies, such as malaria and sickle cell disease. Similarly to albumin, whether the protective effect of hemopexin is explained exclusively by its heme binding capacity remains to be established.

8.4 Heme Transporters

Targeting heme transporters may be of therapeutic relevance. For instance, the induction of heme exporters such as ABCG2 may be used to promote cellular heme secretion while the inhibition of heme importers such as FLVCR1 might prevent heme cellular uptake (**see section 4.2).** However, this therapeutic application requires a better understanding of the heme transporter mechanism(s).

8.5 Therapeutic Application of Antibodies, Abs

A possible therapeutic alternative to prevent heme cytotoxicity is the development of antibodies (Abs) that target “free heme” specifically. These may be applied to neutralize the pro-oxidant and pro-inflammatory effects of heme and hence minimize its participation in the pathogenesis of immune mediated inflammatory diseases associated with hemolysis. This type of therapy has several advantages, such as specificity of Abs towards “free heme”, instead of targeting heme in hemoproteins, thereby not impairing their biologic function.

The therapeutic administration of antibodies begun in 1986 with the first approval for the use of an anti-CD3-specific mAb (Orthoclone OKT3; Janssen-Cilag) by the US Food and Drug Administration, for acute transplant rejection treatment in humans (Emmons and Hunsicker, 1987;Leavy, 2010). Since then several Abs were developed and used therapeutically against cancer, autoimmunity and inflammatory diseases (Elvin et al., 2013). Furthermore, Abs are also widely used in medical diagnostics (Ellmark et al., 2008). The development and production of monoclonal Abs (mAbs) was made possible by the implementation of the hybridoma technology (Yagami et al., 2013), based on mouse (alternatively rat and more recently rabbit immunization and generation of immortalized Ab-producing B cells, known as hybridoma (**Fig. 14**).

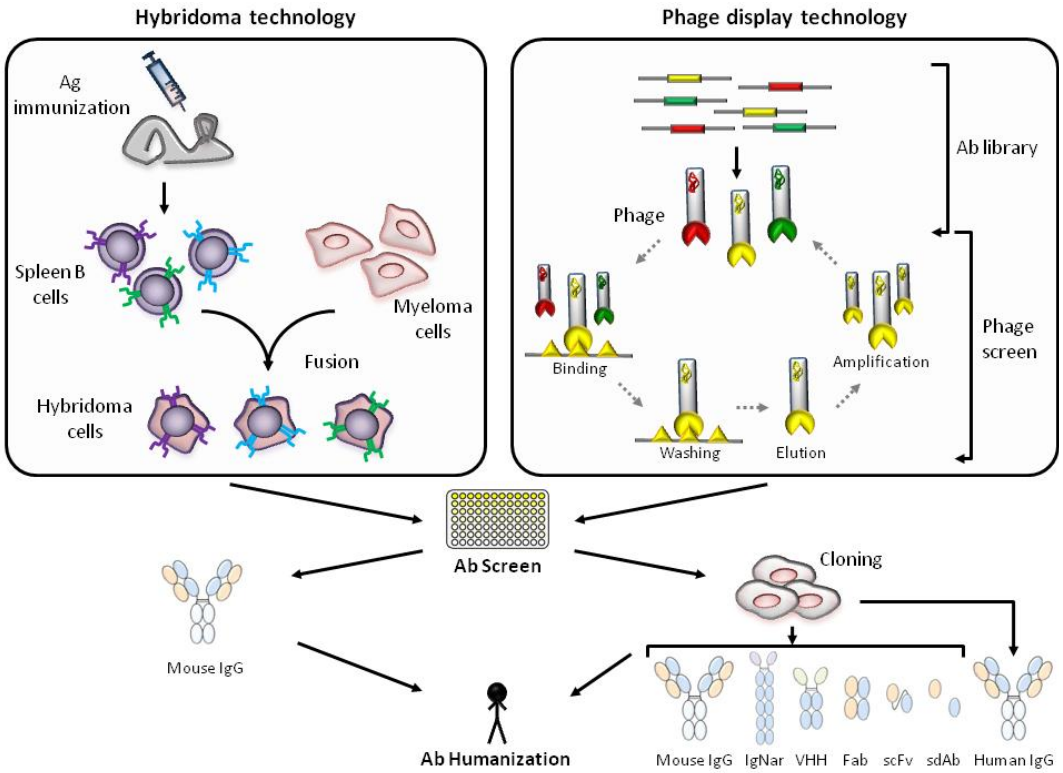
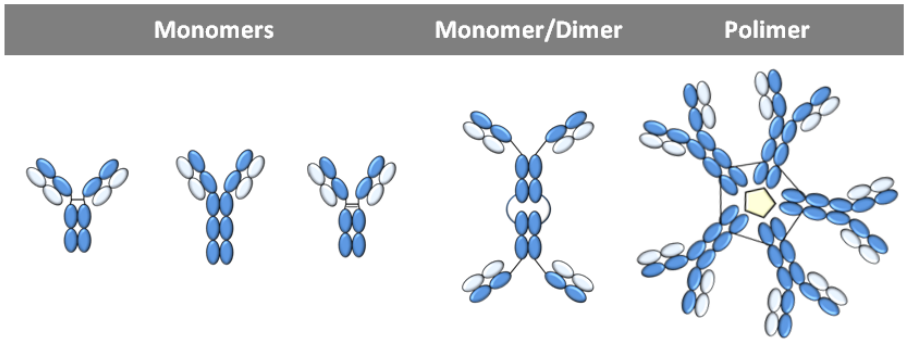


Figure 14: Hybridoma and Phage display technology for the development of Abs. In traditional hybridoma technology, a mouse is immunized and B cells are harvested and immortalized by fusion with myeloma cells to generate hybridomas. These are tested for their ability to produce Abs against the Antigen used in the immunization. Phage display technology amongst other display techniques (Ribosome or bacteria, yeast and mammalian cells) is used in the development of conventional antibodies as well as different constructed Ab (**Fig. 18**). The Abs can be selected from in constructed immune, naïve or synthetic libraries (human, mouse, rabbit, shark or camel), usually with a highly diverse Ab repertoire (10^6-10^{12}variants) displayed in the phage surface. Selection against a given antigen is performed using immobilized antigen in cycles in which the non bounding Abs are washed away and the binding Abs are amplified by infection of *E. coli* bacteria. After several cycles, the selected Abs are screened for Ag specificity. These can be humanized by cloning of the CDR genes into a whole human IgG expression vectors for the production of the fully length human Ab.

8.5.1 Abs Structure and Function

Abs or Ig (s) are high molecular weight plasma proteins (around 150 kDa) produced by B cells and recognizing a vast repertoire of antigens (Ags) (Davies et al., 1990).



	IgD	IgE	IgG	IgA	IgM
Size (*kDa*)	180	200	150	320 (secretory)	900
Serum (mg/mL)	0 - 0.4	0.01 - 0.4	10 - 16	1 - 4	0.5 - 2
Serum (%)	<0.5	<0.01	75	15	10
Main Function	Homeostasis	Allergy	Secondary response	Mucosal response	Primary response

Figure 15: Schematic representation of the five different classes of Abs (IgA, IgD, IgE, IgG and IgM) and some of their basic properties (Table). The different classes of Abs confer protection to the organism against foreign antigens to which they are constantly exposed. While having a similar structure, these Abs differ in the immunological function. The monomers IgD can act as antigen receptor for B cells and activators of mast cells and basophils, inducing the production of antimicrobial molecules, while IgE is involved in host protection against parasitic worms and stimulation of the histamine release by mast cells and basophils. The most abundant Ig in plasma is IgG providing protection against innumerous pathogens, mainly through the activation of the complement system and phagocytosis. This Ig is also capable to cross the placenta of a mother into the fetal circulation providing protection to the fetus. IgA can be mainly found as a monomer in serum and as a dimer in the secretions and is the most common Ig in external secretions, such as mucous, saliva, breast milk and tears, providing protection against invading pathogens. IgM is predominantly found in pentameric form, typically secreted into serum by plasma cells, although it can be expressed on the B cells surface as a monomer. This Ig is also involved in the protection against the pathogens in the beginning of humoral immune response.

There are five Abs isotypes in mammals: IgA, IgD, IgE, IgG and IgM, with different effector functions (**Fig. 15**). IgGs are the most abundant in plasma while IgA is more abundant in the intestinal tract. Abs have a "Y" shape, comprising a constant (Fc) fragment region containing two heavy (H) chains with two or three constant domains, depending on the isotype, linked *via* disulfide bonds to two Ag binding fragments (Fabs) composed by one heavy (H) and light (L) variable (V) domains (also named variable fragment; Fv) and one light and heavy constant (C) domain (**Fig. 16**) (Jakob et al., 2013). The Fv region is essential for Ag recognition and binding, while the Fc region regulates the immune response *via* interaction with a specific class of Fc receptors as well as with others immune components, *e.g.* complement proteins (Heyman, 1996). The Fc region modulates the half-life of the Ig, presumably by its interaction with the Fc receptor, which controls Ig catabolism (Hinton et al., 2004). The variable domains of IgG are composed by three loops containing a hypervariable sequence in amino acid composition and size, named complementarity-determining regions (CDR), present on variable H and L chain. These determine Ab specificity for a given Ag*, i.e.* recognition as well as binding affinity (*i.e.* K_D; binding affinity constant) (North et al., 2011). These CDR loops are inserted in the framework region (FR) that is primarily associated with β-strands conformation, providing an essential structural support, which helps in the preservation of Ig affinity and specificity (Masuda et al., 2006).

Abs can act in two different manners: through direct binding to the pathogen for their neutralization or indirectly *via* the activation of the complement cascade and/or *via* Fc recognition by effector immune cells. The activation of complement cascade, *i.e.* the classical complement pathway, occurs when the Ab bound to the pathogen is recognized through its Fc region by the complement component C1q molecule, triggering a series of events that lead to the generation of opsonins, anaphylatoxins, chemotatic agents as well as the generation of a membrane attack complex that kills pathogens (Diebolder et al., 2014). On the other hand, the Ab-mediated activation of effector immune cells, *e.g*. mast cells, neutrophils, natural killer cells and Mø, occurs when an Ab is recognized through Fc receptors (FcγRs), promoting phagocytosis and killing of pathogens (Guilliams et al., 2014).

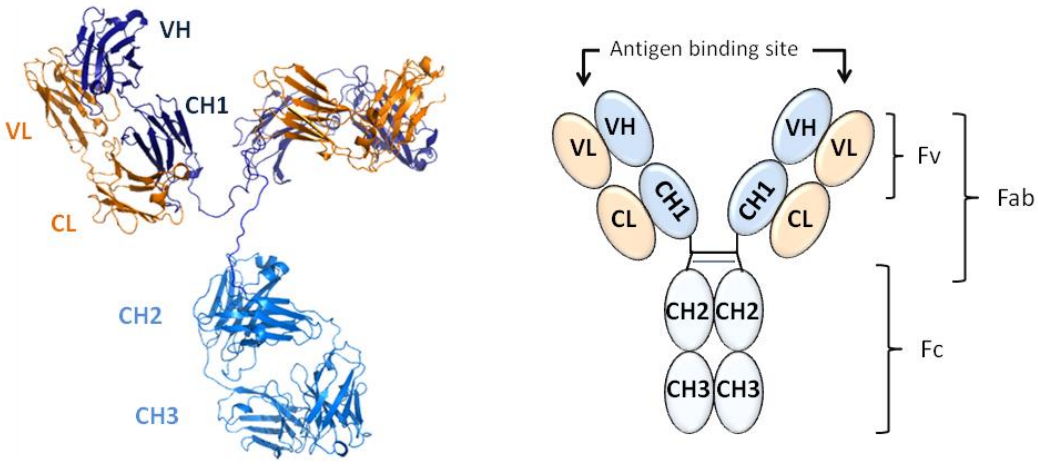


Figure 16: Structure of IgG and its schematic representation. Structure of a anti-canine IgG2a antibody (Mab231) (PDB ID: 1IGT) as representative of a typical "Y" shape form of Ig (left) and its schematic representation (right). IgG are composed by constant (Fc) and an antigen binding fragment (Fab) region. The Fc region is composed by two heavy chain formed by the constant heavy domains 2 and 3 (CH2 and CH3) and it is linked to the Fab region *via* disulphide bonds between the CH2 of Fc and the constant heavy domains 1 (CH1) of Fab. In Fab region beside the CH1 there is a variable heavy domain (VH) and a light chain composed by a constant (CL) and variable domain (VL). The variable domains, VH and VL together form the variable fragment (Fv) and are responsible for the antigen recognition and binding. The Fc region of the Ig controls immune response via interaction with a specific class of Fc receptors or with complement proteins.

8.5.1.1 Ab Fragments

Abs used for therapeutics are in most cases full length IgG, which are also the most abundant in blood and have longer serum half-life as well as the ability to activate complement as well Fc-dependent immune responses. However, IgG Abs can also lead to the inappropriate complement activation and Fc mediated responses associated with production of cytokines, which might trigger uncontrolled inflammation (Chames et al., 2009). Furthermore, the relative size of the Ab does not allow for their removal through the kidneys, which has a clearance threshold of around 40-60 kDa. Additionally, full-length Abs have a low diffusion rate, that might compromise tissues and the extracellular matrix access. For that reason, Ab

fragments are used as an alternative to overcome such limitations, as they have lower molecular sizes while maintaining their ability to bind antigens. These include the monovalent Ab fragments (Fab, single chain Fv; scFv) and engineered variants (diabodies, triabodies, minibodies and single-domain Abs)(**Fig. 17**) (da Silva et al., 2008).

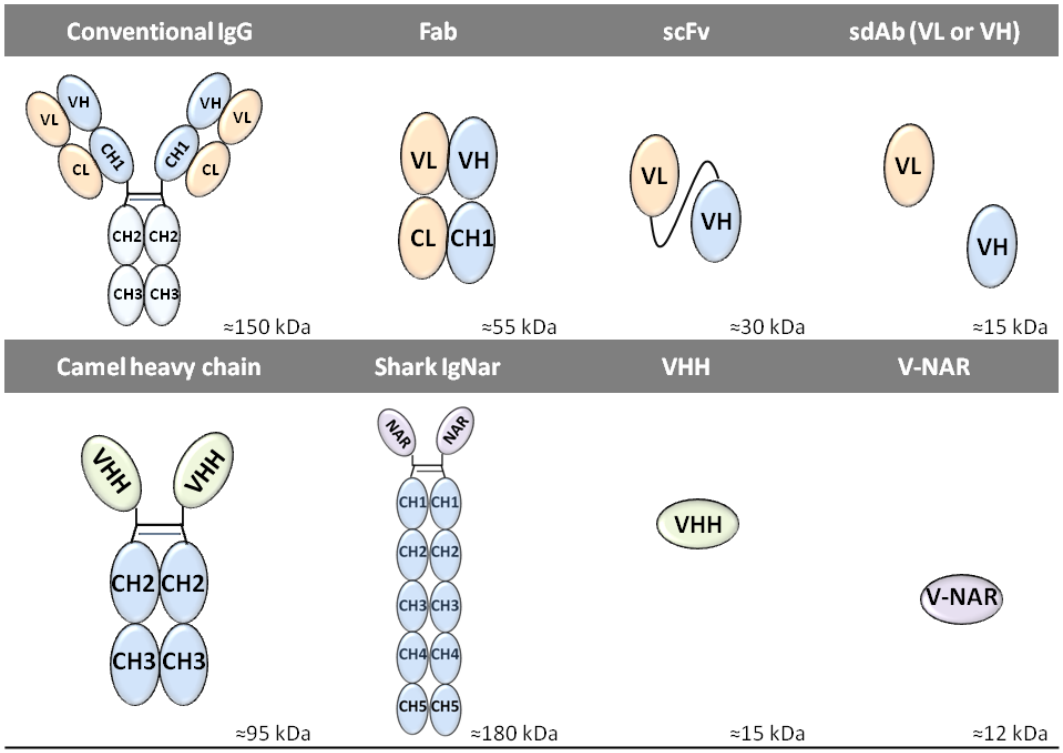


Figure 17: Schematic representation of different types of Abs. Fabs and Fv are based on the Fab and Fv domains of the Ab, respectively while sdAb VL or VH are generated from the VL or VH domains, respectively. The camelid/shark Igs contain only the heavy chain. These include the camel heavy chain antibody composed by two C like constant domains (CH2 and CH3) and a homodimer of the variable heavy domain (VHH) and the sharK IgNar antibody with a homodimer of a variable domain (NAR) and five constant heavy domains (CH1 to CH5) (lower). Other variants include the VH domain (VHH) and variable NAR domain (V-NAR) from camel and shark antibodies, respectively (lower).

8.5.1.2 Single Domain Abs

Single domain Abs (sdAbs) have small sizes (12 to 15 kDa) and are usually composed by a VL or VH single domain, retaining their Ag binding capacity (**Fig. 17**). However, sdAbs have a high tendency for aggregation, mainly due to the exposure of the hydrophobic interface. This drawback can be overcome though genetic engineering, reducing their hydrophobic exposure and increasing stability (Davies and Riechmann, 1996). Alternatively, this can be achieved through generation of dimers *via* the introduction of a flexible linker between two sdAb, *e.g.* VH and VL, a methodology also used to produce scFv (Holliger et al., 1993).

The discovery of functional and highly stable VH sdAbs in camelids (camels, dromedaries and lamas) (Hamers-Casterman et al., 1993) and cartilaginous fish (wobbegong and nurse chark) (Greenberg et al., 1995), suggests that sdAbs engineered to mimic these natural sdAbs have a high potential for biotechnological and eventually therapeutic applications. In fact, sdAbs derived from camelids are widely used in biotechnological applications, because they are easily manipulated and can be obtained through animal immunization (van der Linden et al., 2000) or by other display technologies (Gronwall and Stahl, 2009), such as phage (Binyamin et al., 2003), yeast (Scholler, 2012), ribosome (Yau et al., 2003) or mRNA display (Tabata et al., 2009), using immune (Kim et al., 2008), naïve (Wen et al., 2007) and semi-(de Kruif et al., 1995) or synthetic (Silacci et al., 2005) Ab libraries.

SdAbs provide several advantages in comparison to the full-length Abs and other conventional Ab' fragments (Holt et al., 2003). For example, sdAbs have a higher capacity to reach a wide variety of antigens, including small molecules (Spinelli et al., 2000), as well as specific epitopes in high molecular weight proteins (Arbabi Ghahroudi et al., 1997), interacting specifically with the active site of enzymes, inhibiting their activity (Desmyter et al., 2002). Furthermore, sdAbs penetrate into tissues and are rapidly cleared from the blood through the kidney. Another considerable advantage is that they allow for rather easy genetic manipulation.

The therapeutic application of sdAbs have been explored with some promising results, for instance against cancer and immune mediated diseases, including

infectious diseases (da Silva et al., 2008). The potential targets of the sdAbs are mainly cell surfaces, secreted and intracellular proteins and cytokines, as well as bacterial components.

8.5.2 Ab Production using Phage Display

In 1985, George P. Smith described for the first time the phage display (**Fig. 14**), a technique based on the fusion of foreign DNA sequences into the gene III of the phage, thus promoting the expression of the coated protein pIII, localized in the surface of the virion of the filamentous phage (Smith, 1985) (**Fig. 18**). Interestingly, the fusion of foreign DNA sequences does not change the infectious properties of the phage, while the respectively encoded ("foreign") protein is simultaneously expressed and linked to the coated protein pIII on the virion surface. Through the insertion of a given gene encoding a peptide or protein of interest, *e.g.* Abs, into a phage it is possible to express those in the phage surface. This technology has been explored and developed for screening and selection of a wide variety of Abs, such as fragment Abs, with potentially therapeutic relevance (Hairul Bahara et al., 2013). Thus, phage display technology is currently recognized as a very powerful technique for the selection of Abs (da Silva et al., 2008). It allows to generate phage libraries with a highly diverse repertoire, in the range 10^6-10^{12} variants (Tikunova and Morozova, 2009), that can be applied for screening and selection based on the detection of Ags of interest (Tonelli et al., 2012), including biological as well as inorganic compounds (Kriplani and Kay, 2005). On the other hand, phage display technology also allows for genetic manipulation of the libraries and consequently, for post- selection improvement (Winter et al., 1994).

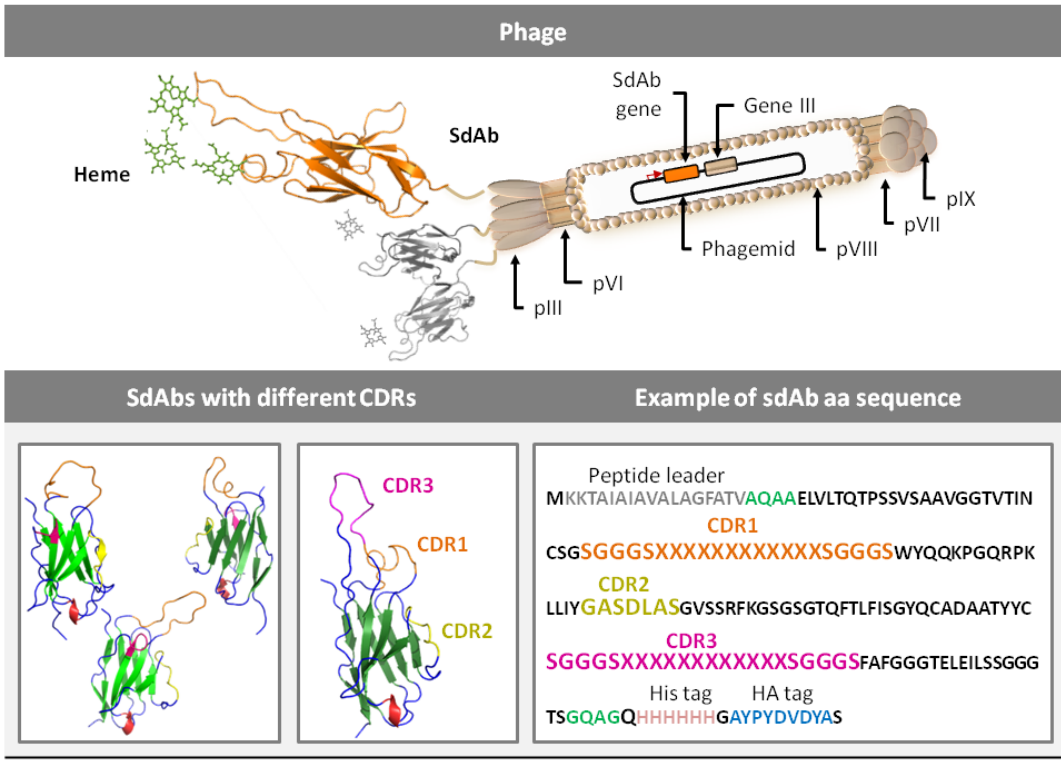


Figure 18: Schematic representation of a filamentous phage expressing sdAb on their surface fused with pIII protein and the structure and amino acid sequence of sdAbs. The filamentous phage (*upper*) displayed in its phagemid, the DNA sequence of the sdAb (in orange) fused into the gene III that codifies for the coated protein III and both genes are expressed from a common promoter. SdAb is expressed fused with the coated protein pIII on the surface of the phage, making accessible to the sdAb the target molecule, such as heme. Besides the coated protein pIII, there are other coated proteins such as pVI, pVII, pVIII and pIX that can also be used to display foreign proteins/Abs. 3D structure of three sdAbs, namely VL, generated based on the aa sequence of the anti-heme sdAbs 1A6, 2H7 and 2H10 using SWISS-MODEL software (http://swissmodel.expasy.org/interactive)(*lower, left*). Crystal structure obtained for a human sdAbs VL (PDB ID: 3LRG) against the huntingtin protein (*lower, middle*). An example of an aa sequence encoding a rabbit based VL, containing a starting amino acid (M), a peptide leader (gray) that conduct the new synthesized protein towards the secretory pathway, two restriction enzyme site (green; SfiI enzyme), the framework of the Ab (black) with the CDR1 (orange), CDR2 (yellow) and CDR3 (pink) and a Histidine (light pink) and HA tag (blue) that are incorporated in the sdAbs for its detection and capture (*lower, right*). The CDR1 and CDR3 contain a SGGGS followed by n number of X which indicates that each of them can be any of the 23 amino acids, conferring variability amongst the sdAbs of phage display library.

8.5.2.1 Selection and Screening of Abs using Phage Display Technology

The screening and selection of Abs, *e.g.* sdAbs, using phage display technology is dependent on the efficacy of inserting and maintaining the gene encoding the protein of interest into the phage genome, as well as the phage capacity to express that protein on its surface. Therefore, an intrinsic connection between the genotype and the phenotype of the expressed protein should be taken into consideration, as to allow the enrichment of a phage population containing the protein of interest during selection (Li and Caberoy, 2010). The selection process for sdAbs can be divided in several steps, comprised into a cycle (**Fig. 19**) (Frenzel et al., 2014):

1st step: Ag coating onto a solid or liquid phase;

2nd step: exposure and incubation of a phage library repertoire with the Ag, *i.e.* phages that express the Abs are allowed to bind specifically to the Ag;

3rd step: Removal of unbound phages through a series of wash-off rounds;

4th step: Elution of Ag-bound phages using methods allowing for the disruption of the ligation between the displayed Ab and Ag;

5th step: Re-amplification of the eluted phages by infection into host bacterial cells.

Generally, this cycle is repeated three to six times for the generation of Abs with high affinity and specificity for a given Ag. Nevertheless, the quality of the Abs selected is highly dependent on the characteristics of the phage library used, such as repertoire diversity, quantity and affinity (Hoogenboom, 1997;2005).

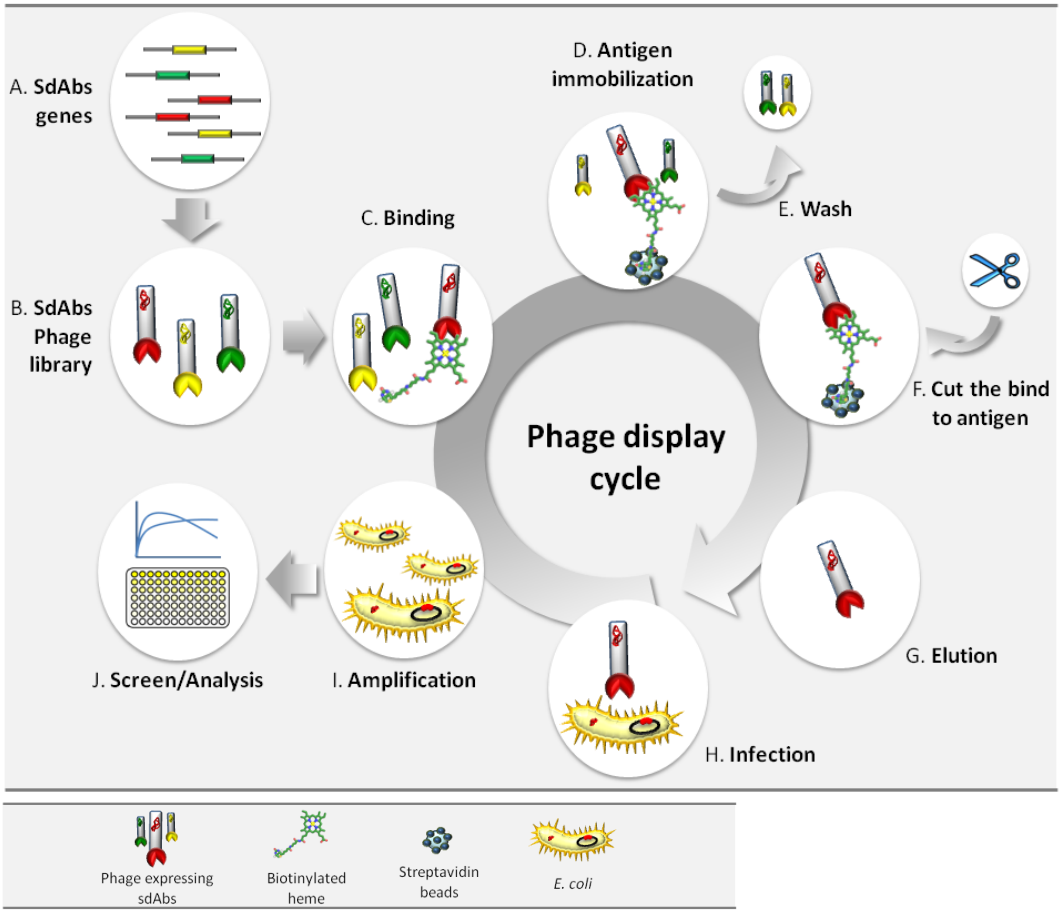


Figure 19: Schematic representation of a phage display cycle, also named phage display panning, applied in the selection of Abs. A phage display library is constructed through the generation of **A.** a library containing a high content of variant DNA sequence encoding proteins (10^6-10^{12} variants), namely Ab that is **B.** fused into the gene encoding a phage coat protein in the phagemid genome of the phage, allowing the expression of the protein of interest linked to the phage coated protein (**Fig. 16**). **C.** The phage display library is exposed to an Ag (such as heme) and the phages expressing the Abs with the appropriated specificity are captured. There are several methods for the immobilization of the target. In this work, we biotinylated the target *i.e.* heme (**C.**) and **D.** immobilized it using streptavidin beads after exposure with the phage library. **E.** A series of wash-off rounds is applied to remove non bound Abs-phage. **F.** The ligation between the phage displaying the Ab and the antigen are disrupted by enzymatic digestion (*e.g.* tripsin). **G.** After the disruption, phages are eluted and **H.** used to infect host bacterial cells (*e.g. E.coli*) for **I.** phage amplification. Generally, this cycle is repeated three to six times, decreasing the phage diversity while increasing affinity and specificity for the target. After several cycles, the individual phages from the selected phage population are **J.** screened and analyzed for the binding to the target.

8.5.2.2 Libraries used in Phage Display Technology

The success of Ab selection using phage display technology relies essentially on the quality of the Ab library. Therefore, several types of phage Ab libraries, with high degree of protein variance have been constructed, including immune (Kim et al., 2008), naïve (Wen et al., 2007) and semi- (de Kruif et al., 1995) or synthetic (Silacci et al., 2005) libraries.

The immune libraries are constructed based on the sequence acquired from the IgG mRNA from B cells, obtained through different lymphoid sources (peripheral blood, bone marrow, tonsils lymphoid nodes and, predominantly, from spleen) of immunized donors (McCafferty et al., 1990). Usually, these libraries are enriched in Abs with a higher affinity and specificity for a specific Ag (Winter et al., 1994), including virus components (Barbas et al., 1992b), bacterial and other derived toxins (Pelat et al., 2009) as well as tumor antigens (McWhirter et al., 2006;Siva et al., 2008). Nevertheless, several limitations are associated with these libraries, such as i) long waiting periods due to donor immunization, ii) limited availability of human B cells which limit human Abs generation, iii) restricted or absent immune response to certain Ag (self or toxic), iv) uncertainties in the immune response against the target Ag and, most importantly, v) it is required to generate a new library for each Ag (Hairul Bahara et al., 2013).

The generation of naïve libraries does not require immunization; instead it is constructed based on rearranged V genes from IgM mRNA of B-cells from healthy donors (Sommavilla et al., 2010). The choice of IgM instead of IgG is mainly due to the lack of somatic mutation in V genes of naive and generally IgM (Bradbury and Marks, 2004). Furthermore, previous experiments demonstrated that certain Abs could only be isolated from IgM-and not from IgG-based libraries (de Haard et al., 1999) and since then the libraries used are essentially IgM-based (Sblattero and Bradbury, 2000). The main advantage of these libraries is the possibility to use the same library for the Abs screen against a wider range of antigens (Hairul Bahara et al., 2013). However, the success of such screen is highly dependent on the size and diversity of the naïve libraries (Marks and Bradbury, 2004). Moreover, these libraries can be used to obtain human Abs against self, non-immunogenic, toxic

antigens or chemical haptens (Griffiths et al., 1993;Hairul Bahara et al., 2013). On the other hand, most of the Abs selected in naïve libraries exhibits lower or moderate affinity for the Ag in comparison to those selected in immunized libraries, and little or no information exists about the exact nature of the V-gene repertoire used to generate these libraries. Moreover, because the genetic information generated about the Abs is representative of the human immunological repertoire, there is a high probability that gene expression and protein stability in the bacteria are compromised (Swartz, 2001). Thereby, several methodologies have been developed to overcome such limitations, including improvements in bacterial expression systems (Rouet et al., 2012) or rearrangement of the V-genes from germline B cells (Griffiths et al., 1994) or from a single germline Ab segment (Pini et al., 1998) through CDR randomization, resulting in a semi-synthetic library.

Semi-synthetic libraries are thus generated by the combination of naïve and synthetic derived CDR repertoires, in which the variations are restricted to the CDR regions, while the main framework is obtained from the naïve library (Hoet et al., 2005). Contrastingly, synthetic libraries are usually generated based on a specific (*e.g.* human, rabbit) derived framework with randomized synthetic CDR regions to provide Abs diversity (Nahary and Benhar, 2009). Among the three CDRs (**Fig. 18**), heavy chain CDR3 (CDR-H3) is used predominantly for randomization to increase diversity, since it is the main contributor for Ab-Ag binding interaction (Barbas et al., 1992a). This randomization of the CDR-H3 follows certain rules (Shirai et al., 1999), used to determine the tertiary structure of the CDR, particularly important upon substitution of a specific amino acid, which might comprise the Abs structure (Kuroda et al., 2008). Apart from modifications in aa sequence composition and consequently structure, the sequence size itself also generates diversity (Oliva et al., 1998;Almagro, 2004). These, however, were revealed not to be an exclusive property of the CDR-H3, but also of other CDRs, as demonstrated upon the discovery of residues directly involved in the recognition of the Ag, named specificity-determining residues (Almagro, 2004). Therefore, through the randomization of key residues in the CDRs, it is possible not only to increase the diversity of the library, but also to select Abs with higher affinity (Huls et al., 1999),

as demonstrated for the human combinatorial Ab library, a full synthetic library (Knappik et al., 2000). The main advantages of the synthetic libraries rely on the fact that they easily allow for generating highly diverse libraries without requiring a natural immune repertoire. Moreover, the Ab frameworks can be chosen to improve the stability and expression yield accordingly to the expression system and to prevent Abs driven immunogenicity. These libraries also retain some of the characteristics of the naïve libraries, such as multi-Ag targeting by one library alone. However, there are still some limitations, such as the higher frequency of unnatural amber stop codons and glycosylation sites during the scFvs randomization process, which may compromise the structure and specificity of the Ab (Solforosi et al., 2012).

9 AIM OF THE THESIS

The research work presented in this PhD thesis was developed to overcome the current limitations in the detection and quantification of “free heme”, once released from hemoproteins. For that purpose, we developed sAbs, using phage display technology that recognizes specifically heme that is not bound to hemoproteins. These sdAbs were characterized for their specificity to bind heme and selected according to their binding specificity and affinity. The selected sdAb were then further characterized and tested for their ability to recognize cellular heme, to provide a quantitative detection of “free heme”, as well as to function as modulators of heme biological activity.

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CHAPTER 2

ARTICLE:

Targeting Heme with Single Domain Antibodies

Targeting Heme with Single Domain Antibodies

Zélia Gouveia[1,4], Ana Rita Carlos[1], Frederico Aires-da-Silva[2,9], Roland Stocker[3], Ghassan J. Maghzal[3], Sónia S. Leal[4], Cláudio M. Gomes[4], Smilja Todorovic[4], Olga Iranzo[4,£], Ana Catarina Santos[6,7], Xiaojing Yuan[5], Iqbal Hamza[5], João Gonçalves[6,7] & Miguel P. Soares[1,a*]

[1]Instituto Gulbenkian da Ciência, Portugal.; [2]Technophage S.A., Portugal.;[3]Vascular Biology Division, Victor Chang Cardiac Research Institute, Australia; University of New South Wales, NSW, Australia.; [4]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal.; [5]Department of Animal and Avian Sciences and Department of Cell Biology and Molecular Genetics, University of Maryland, , USA.; [6]CPM-URIA Faculdade Farmácia, Universidade de Lisboa, Portugal.; [7]IMM, Faculdade de Medicina, Universidade de Lisboa, Portugal.; [h]CIISA-Faculdade de Medicina Veterinária, Universidade de Lisboa, Portugal.

[£]Current address of OI: Aix Marseille Université, Centrale Marseille, CNRS, iSm2 UMR 7313, 13397 Marseille, France

*To whom the correspondence should be addressed: Miguel P. Soares; Inflammation Group, Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal, Tel.: +351-214464520;

E-mail: mpsoares@igc.gulbenkian.pt

Manuscript under submission

Keywords

Heme, hemoglobin, reactive oxygen species (ROS), stress, hemoproteins, hemolysis, immune mediated diseases, inflammation, antibody engineering, single domain antibodies,

Author contribution

ZG designed and performed all the experimental work and analyzed the data with the exception of ascorbate oxidation designed and performed by RS as well as immunofluorescence and Flow cytometry assays designed and performed by ARC. JG and FAS conceived and designed the synthetic phage display library and contributed critically to screening of sdAbs by Phage display technology. CMG and SSL contributed with the analysis by Circular Dichroism and Attenuated total reflectance Fourier transform infrared spectroscopy. ST and ZG performed Resonance Raman analysis. OI and ZG performed heme biotinylation. IH and XY developed the Golgi-HRP assay and assisted ZG; MPS designed and supervised the study wrote the manuscript with ZG. All the authors read, corrected, comment and approved the manuscript.

1. ABSTRACT

Heme acts as a prosthetic group in a number hemoproteins that support core biologic functions essential to sustain life. When exposed to different forms of stress hemoproteins that bind heme non-covalently, can release their heme groups. This produces “free heme”, which acquires unfettered redox activity as it binds loosely to other proteins or molecules. The “free heme” generated in this manner exerts pro-oxidant, pro-inflammatory and cytotoxic effects that contribute critically to the pathogenesis of immune mediated inflammatory diseases, associated with hemolysis. There are current methodological limitations that restrain the qualitative and quantitative analyzes of “free heme” as it is generated in the context of these pathologic conditions. Some methods have been developed to access the presence of “free heme”, however, the controversy has been raised regarding the precision of those methods to detect and quantify it. To overcome this hurdle, we have generated and characterized a panel of single domain antibodies (sdAb) recognizing specifically “free heme”. We demonstrate that heme binding by these sdAbs requires the iron atom contained in the protoporphyrin ring, as they fail to recognize other related tetrapyrrole structures from which the iron was released such as biliverdin or bilirubin. Qualitative and quantitative analyzes of cellular “free heme” using these sdAbs, reveals that cellular “free heme” exists essentially in the mitochondria and to some extent in the cytoplasm, where “free heme” is in close association with the heme catabolizing enzyme heme oxygenase-1. We used these sdAbs to develop ELISA based assays that allow for qualitative and quantitative characterization of “free heme” as it accumulates in plasma during acute hemolytic conditions. Finally we demonstrate that binding of these sdAbs to “free heme” in solution reduces heme redox activity, potentially therefore overcoming some of the pathologic effect exerted by “free heme”. In conclusion, our findings contribute to overcome the current limitations in the quantitative assessment of “free heme” under different biologic contexts and allow targeting “free heme” therapeutically to overcome pathologies associated with hemolysis.

2. INTRODUCTION

Heme consists of a tetrapyrrole ring bound via different nitrogens to a central iron (Fe) atom (Fischer and Zeile, 1929). Heme is found essentially as a prosthetic group of hemoproteins (Tsiftsoglou, 2006), including some that sustain core biologic functions essential to support most if not all life forms. Specific alterations in the vinyl and methyl group composition of the tetrapyrrole ring define different types of heme, namely, heme *a* ($C_{49}H_{56}O_6N_4Fe$), *b* ($C_{34}H_{32}O_4N_4Fe$) and *c* ($C_{34}H_{36}O_4N_4S_2Fe$). Heme *a,* associates to hemoproteins, such as cytochrome c oxidase (Tsukihara et al., 2003), *via* hydrogen bonds between the tetrapyrrole formyl and hydroxyfarnesylethyl groups and different amino acid (aa) residues such as arginine and serine. The less abundant heme *c* binds covalently to cysteine via its vinyl groups, as illustrated for cytochrome c (Bushnell et al., 1990). Heme *b* is the most abundant form of heme in mammals and associates to proteins *via van der Waals* contacts formed between heme-face and different hydrophobic aa, as well as through electrostatic interactions with positive side chains and the negative charged propionate groups of heme via salt-bridges (Schneider et al., 2007). Heme *b* is found essentially as a prosthetic group of hemoglobin (Hb) and myoglobin (Mb), accounting for the major pool of bioavailable Fe in mammals (Hunt, 2003).

The Fe contained within the tetrapyrrole ring of different types of heme can bind to histidine, methionine and lysine, which control Fe redox status and stabilize heme/hemoprotein binding and activity. Moreover, modification of heme Fe oxidation state, *i.e*. ferrous (Fe^{2+}), ferric (Fe^{3+}) or higher oxidation states up to Fe^{5+}, also regulate heme/hemoprotein binding and activity (Chapman et al., 1997). The redox active Fe in the heme group of those hemoproteins provides optimized binding to gaseous molecules such as O_2 as well as gasotransmitters (Mustafa et al., 2009) such as nitric oxide (NO) (Moncada and Erusalimsky, 2002) or carbon monoxide (CO) (Dioum et al., 2002), *via* effective back-donation of Fe $d\pi_x$ electrons to low-lying π^* orbitals of these diatomic molecules. Hemoproteins can fine-tune this interaction in order to modulate i) catalysis of reductive and oxidative chemistry, ii)

storage and/transport of gaseous molecules, or iii) signalling processes underlying the biologic activities of gasotransmitters (Mustafa et al., 2009).

Non-covalently bound heme *b* can be released from hemoproteins (Balla et al., 1993), as demonstrated for Hb when exposed to hydrogen peroxide (H_2O_2) generated by activated polymorphonuclear leukocytes (Bunn and Jandl, 1968;Hebbel et al., 1988;Balla et al., 1993;Pamplona et al., 2007). Non-Hb bound heme is often referred as “free heme” (Ferreira et al., 2008;Larsen et al., 2010). However, considering that heme is probably never found in free form in biologic systems, the term “free heme” is used hereafter to refer to the pool of labile redox active heme that is loosely bound to proteins or to other molecules.

Once released from hemoproteins heme *b* can act in a vasoactive, pro-inflammatory (Graca-Souza et al., 2002;Figueiredo et al., 2007) and cytotoxic manner (Seixas et al., 2009;Gozzelino et al., 2010;Larsen et al., 2010) to promote the pathogenesis of immune mediated inflammatory diseases associated with hemolysis, as illustrated for sickle cell disease (Ghosh et al., 2013;Vinchi et al., 2013;Belcher et al., 2014), malaria (Pamplona et al., 2007;Ferreira et al., 2011) or severe sepsis (Larsen et al., 2010). It has been challenging to ascertain unequivocally the pathologic role of “free heme”, largely due to the current limitations in the available methodologies allowing its quantification and/or providing information on its chemical environment. Here we used a phage display screening to identity single domain antibodies (sdAb) recognizing specifically redox active heme that is loosely bound to proteins or to other molecules, i.e. “free heme”. We describe and characterize a panel of such sdAbs and illustrate their binding properties as well as usage for quantitative detection of “free heme” and modulation of heme redox activity.

3. RESULTS

3.1 Development and selection of sdAbs against "free heme"

We generated a synthetic phage-display single domain antibody (sdAb) (Goncalves, 2008;Santos, 2014) derived from a highly soluble and stable VL scaffold (Goncalves et al., 2002). The synthetic VL library repertoire, consisting of $8x10^9$ independent clones, was generated by randomization, accommodating a maximum of 22 aa in complementary determining region 1 (CDR1) and 3 (CDR3) (Santos, 2014). The library cloned into the pComb3x phagemid vector (Barbas et al., 1992) was previously screened against several targets (Santos, 2014). In the present study this library was screened against heme *b*, biotinylated in a single carboxylic group (**Fig. 1A and Suppl. Fig. 1**). Phages expressing sdAbs recognizing heme-biotin were selected using streptavidin-magnetic beads (**Fig. 1B and 1C**), essentially as described (Barbas, 2001). Phages were then re-selected according to their relative heme binding activity and expression, as assessed by ELISA using a monoclonal antibody (mAb) directed against the human influenza hemagglutinin (HA) epitope (YPYDVPDYA) expressed in the C-terminus of the sdAb. Eleven sdAbs were selected (**Fig. 1C**), expressed in *E. coli* BL21, purified under denaturing conditions and refolded by step-wise dialysis (Umetsu et al., 2003). Protein yield was typically around 10 mg/L with >90% purity, as assessed by SDS-PAGE (**Fig. 1D**). Expression of a ~15 kDa protein corresponding to the full-length sdAb containing the leader peptide sequence (MKKTAIAIAVALAGFATV) and a ~13kDa protein, corresponding to the leaderless sdAb was confirmed by Western blotting (**Fig. 1D**).

The selected sdAbs bound heme with an affinity in the range of 10^{-7}M with the exception of sdAb 1E4, which had an affinity in the range of 10^{-5}M, as assessed by ELISA (**Fig. 1E**) and surface plasmon resonance (SPR; BIAcore system)(**Fig. 1F**)**.** In **Suppl. Fig. 2** is shown a sensogram of heme binding to sdAb 2H10 obtained by BIAcore, used to determine the affinity constant (K_D), as a representative of sdAb binding to heme. *In silico* analyses revealed the presence of potential heme binding motifs (HBM) (Li et al., 2011) in the CDR1 of some sdAbs.

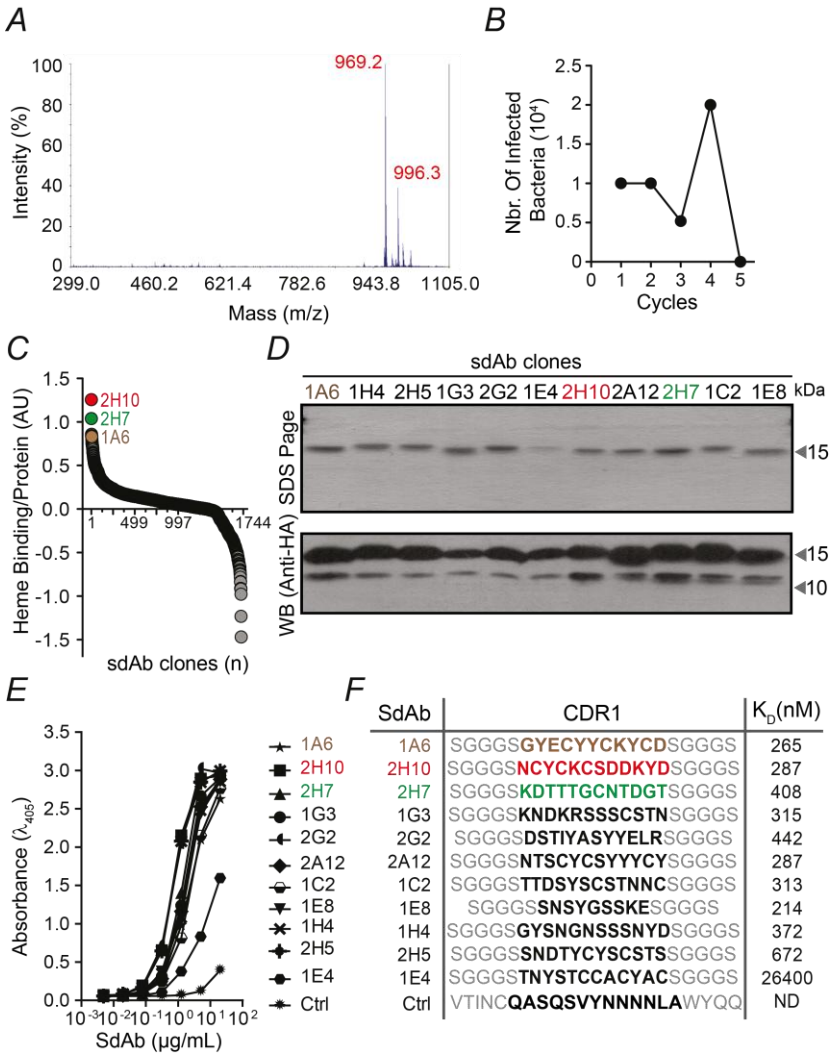

Figure 1. Selection of heme binding sdAbs. (A) MALDI-TOF/TOF analysis of biotinylated heme. Mass-to-charge (m/z) 969.2 Da corresponds to the biotinylation of a single carboxylic acid residue of hemin. (B) Output of elution cycles, selecting bacteria infected with phages displaying sdAbs recognizing heme. (C) Ratio of heme binding *vs.* protein expression, as determined by ELISA. Each circle represents one sdAb among 1721 tested. SdAbs 2H10, 2H7 and 1A6, corresponding to the highest heme binding *vs.* protein expression, are highlighted. (D) SDS-PAGE of purified sdAbs detected by coomassie-based stain or by western blot using an anti-HA mAb. (E) Relative binding of sdAbs to heme detected by ELISA. Control sdAb (Ctrl) does not recognize heme. (F) CDR1 aa sequences corresponding to each sdAbs, as determined by DNA sequence analysis. Binding affinity of SdAbs towards heme, determined by BIAcore surface Plasmon resonance.

These include cysteine-X-X-cysteine-Lysine (CXXCK; were X is any aa) characteristic of hemoproteins binding heme *c* (Pearson et al., 2007), *i.e.* sdAb 1A6, cysteine-asparagine (CN), characteristic of hemoproteins binding heme *b*, *i.e*. sdAb 2H7 or cysteine-serine (CS) also characteristic of hemoproteins binding heme *b*, *i.e.* sdAb 2H10, 1G3, 2A12, 1C2 and 2H5 (**Fig. 1F**). None of the sdAbs selected presented CDR3 sequences. Based on their relatively higher affinity towards heme and the presence of potential heme binding motifs, we focus our analyses on sdAbs 1A6, 2H7 and 2H10.

3.2 Heme sdAb specificity

To determine the specificity of sdAbs 1A6, 2H7 and 2H10 towards heme, we developed an ELISA in which the sdAb were pre-incubated with heme (Iron protoporphyrin; FePP), gallium protoporphyrin (PP) IX (GaPP; a non-iron metalled porphyrin containing a similar sized but redox inert gallium atom) or PP IX (PP; the metal-free precursor of heme). As expected pre-incubation with FePP abolished binding to solid-phase heme by all three sdAbs (**Fig. 2A**). Pre-incubation with PP inhibited heme binding by sdAb 1A6 but not by sdAbs 2H7 and 2H10 (**Fig. 2A**). This indicated that the sdAbs have intrinsic differences in their binding properties and that a metal center is essential for heme binding by sdAbs 2H7 and 2H10. Pre-incubation with GaPP abolished heme binding by all three sdAbs (**Fig. 2A**), indicating that Fe redox state has little or no effect on the capacity of these sdAbs to recognize heme.

Similar results were obtained using tin PP (SnPP) or cobalt PP (CoPP), containing Sn or Co instead of Fe as the metal ion (**Fig. 3A**). Pre-incubation of sdAbs with zinc PP (ZnPP), which is produced physiologically by the heme synthesis enzyme ferrochelatase during iron deficiency, inhibited heme binding by 2H7 sdAb but not by sdAb 1A6 and 2H10 (**Fig. 3A**). The small binding to ZnPP in sdAb 1A6 was considered no significant when compared with the other heme analogs. This might suggest that the presence and the physical/chemical properties of this metal, for example the ionic radii, oxidation state, may be important determinants for heme recognition by the sdAb 2H7. Indeed, it was shown that Zn

is usually coordinated with four ligands in a tetrahedral molecular geometry and it is limited to a redox state Zn2+ which confers some inertiness to the protoporphyrin. This properties of the metal might compromise its protein binding (Pace and Weerapana, 2014).

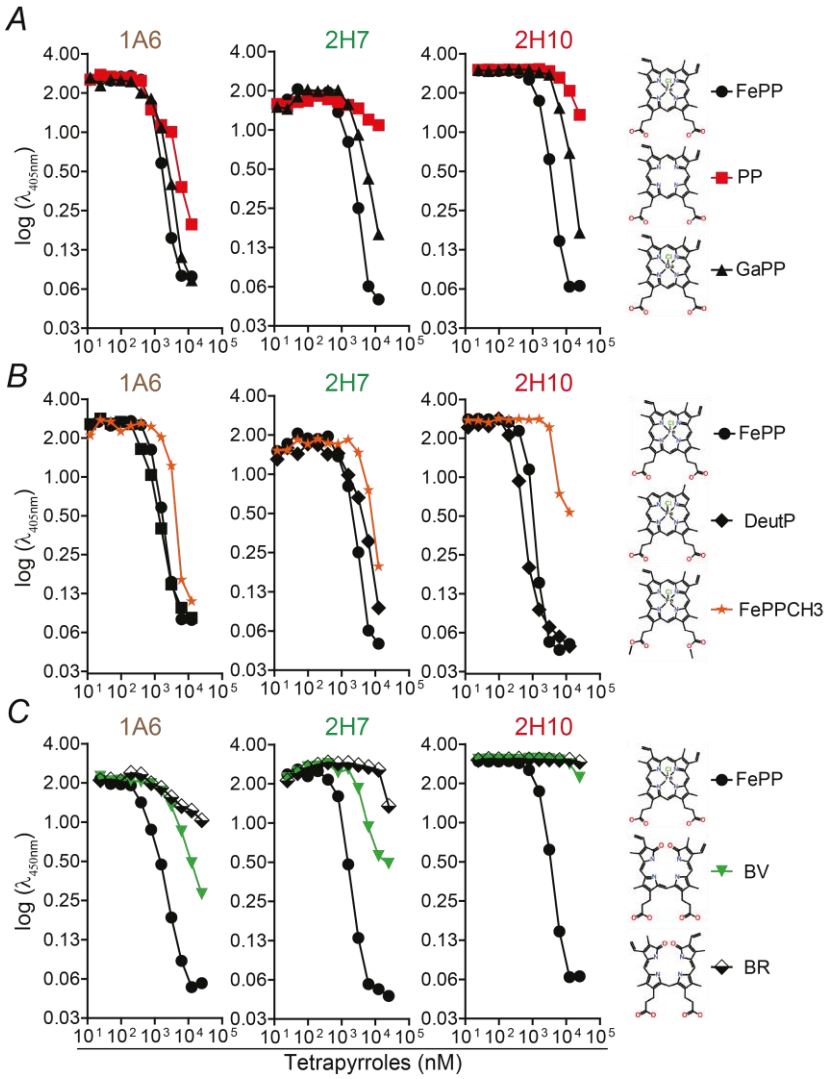

Figure 2. SdAbs heme binding specificity. (A-C) Increasing concentrations of different tetrapyrroles were tested for their ability to interfere with sdAbs binding to solid phase bound heme, as measured by the ELISA. Tetrapyrroles tested include: (A) PP and GaPP, (B) DeutP and FePPCH3, (C) biliverdin (BV) and bilirubin (BR). Hemin (FePP) was used as a positive control. Data shown is representative of three independent experiments.

We verified whether the porphyrin side chains are involved in heme recognition, and pre-incubated the sdAbs with Fe deuteroporphyrin IX (DeutP) or Fe PP IX dimethyl ester chloride (FePPCH3), two synthetic heme derivatives lacking the heme vinyl groups or in which two propionates side chains are methylated, respectively. DeutP inhibited heme binding by all sdAbs 1A6 (**Fig. 2B**), while FePPCH3 prevented sdAb 1A6 and 2H7, but not 2H10 heme recognition. This suggests that heme *b* recognition by sdAb 2H10 involves a carboxylic acid of one of the two propionate side chains (**Fig. 2B**). We also verified whether the sdAbs recognize biliverdin and bilirubin, two tetrapyrrole products of heme degradation by heme oxygenase and biliverdin reductases, respectively. Pre-incubation with biliverdin or bilirubin failed to inhibit heme binding by sdAb 2H10 but not by sdAb 1A6 and 2H7, which failed to recognize heme when pre-incubated with biliverdin but not bilirubin (**Fig. 2C**). This partial inhibition by biliverdin suggests that full inhibition might only by be achieved when this heme end-product is at higher concentration. Thus *in vivo* inhibition of heme binding to these sdAbs can occur when biliverdin is at higher concentration than heme. Nevertheless, further studies are required to address this issue. Iron (III) sulfate (Fe_2 $(SO_4)_3$) failed to inhibit heme binding by these sdAbs (**Fig. 3B**). Pre-incubation of sdAbs with Hb, oxidized hemoglobin, Mb, albumin containing heme *b* or cytochrome c also failed to inhibit heme binding by all three sdAb (**Fig. 3C**). This suggests that, under the experimental conditions used, these sdAbs do not recognize bound heme, as present in hemoproteins. This might be justified by the higher affinity of those hemoproteins for heme when compared with the sdAbs, or heme in those hemoproteins is not accessible to the sdAbs because when incorporated in their heme pocket, it may be shield from the sdAb by the protein structure., We cannot exclude however, that these sdAbs might recognize heme when bound to other hemoproteins, not tested hereby. Table 1 summarizes these results.

These results suggest that each of the sdAbs can recognize heme in a distinct manner, while the sdAb 2H7 and 2H10 required the iron metal for heme recognition, the sdAb 1A6 does not. Besides that heme recognition by sdAb 2H10 involves its interaction with a carboxylic acid group of heme. These allow us to

speculate that the three sdAbs can be used in simultaneous to target different epitopes of heme without competing against each other, which might improve their efficiency to target hem, inhibiting its deleterious effect.

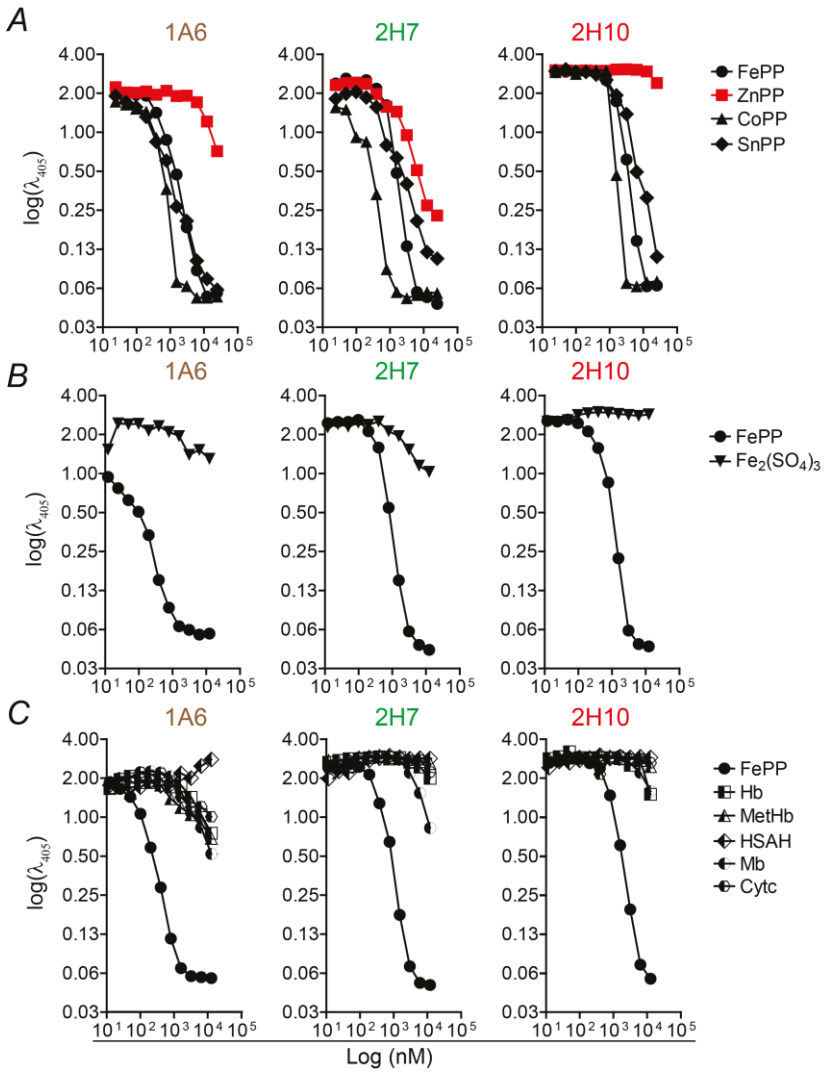

Figure 3. SdAbs heme binding specificity to other heme analogs and hemoproteins. (A-C) Increasing concentrations of different compounds were tested for their ability to interfere with sdAbs binding to solid phase bound heme, as measured by the ELISA. (A) ZnPP, CoPP and SnPP. (B) $Fe_2(SO_4)_3$. (C) Hb, oxidized Hb (MetHb), Mb, Cytochrome c (Cytc) and human serum albumin bound to heme (HSAH). Hemin (FePP) was used as a positive control. Data shown is representative of three independent experiments.

Table 1: Summary of the sdAbs binding specificity against different tetrapyrroles. SdAbs 1A6, 2H7 and 2H10 recognize (+) or not (-) the different tetrapyrroles and some only when at higher concentration (+/-).

	Tetrapyrroles									
sdAbs	FePP	PP	GaPP	ZnPP	CoPP	SnPP	DeutPP	FePPCH3	BV	BR
1A6	+	+	+	-	+	+	+	+	+/-	-
2H7	+	-	+	+	+	+	+	+	+/-	-
2H10	+	-	+	-	+	+	+	-	-	-

3.3 Structural characterization of sdAbs/heme complexes

To determine whether the sdAbs bind heme in solution, we used a heme-biotin pull-down assay. All three sdAbs bound heme in solution, with sdAbs 1A6 and 2H10 showing a higher binding capacity, as compared to sdAbs 2H7 (**Fig. 4A**), which is consistent with the affinities of these sdAbs towards heme (**Fig. 1F**).

We then used UV-Visible spectroscopy to monitor heme (**Fig. 4B**) and heme binding to sdAb 2H10 (**Fig. 4C**). The binding of heme to sdAb 2H10 was associated with the appearance of several bands, including a δ band at 360nm, a very intense Soret band at 412 nm, two weak Q bands at 530 and 565 nm, and a band in the charge transfer region at 624 nm (**Fig. 4C**). Within the heme/sdAb complex, we observed a Soret band at 412 nm (**Fig. 4C**), which indicates the presence of Fe^{3+}. The 530 and 565 nm bands (**Fig. 4C**) reveal a six-coordinated low spin Fe^{3+} state. The δ band at 360 nm (**Fig. 4C**) suggests the presence of a thiol axial heme ligation in the complex (Marvin et al., 2008). The 624nm band is indicative of either S (Cys) to Fe^{3+} charge transfer or porphyrin to high spin Fe^{3+} charge transfer. With the exception of the charge transfer band, UV-Vis features of the sdAb/heme complex were remarkably similar to the spectra of ferric cytochrome P450cam, which carries Cys and H_20 axial ligands (Mouro et al., 1997). Similar results were obtained for sdAbs 1A6 and 2H7 (**Suppl. Fig. 3A and 3B**). The UV-Vis spectrum of heme (**Fig.**

4B) shows two overlapping bands, at ~ 363 and 383 nm, in the Soret region and a charge transfer band at 622, indicating high spin Fe^{3+} state.

Alterations in the secondary structure of sdAb 2H10 upon heme binding were ascertained by circular dichroism (CD) and Attenuated total reflection Fourier Transform infrared (ATR-FTIR) spectroscopies. The far-UV CD spectrum of sdAb 2H10 revealed a negative band centered at 218 nm, indicative of a β-sheet protein fold. Heme ligation resulted in broadening of this band, suggesting a conformational rearrangement of secondary structure elements upon cofactor binding (**Fig. 4D**). Analysis of the Soret region of the spectra reflects heme coordination via appearance of a positive CD band at 412 nm, absent in apo-2H10 sdAb (**Fig. 4D, inset**). The impact of heme coordination on the secondary structure of sdAb 2H10 was further analyzed by ATR-FTIR, which is sensitive to different types of β-sheet structures. A comparison of the absorption spectra of sdAb 2H10 in the presence/absence of heme, in the amide I region (1700-1610 cm^{-1}) is also indicative of structural alterations upon heme coordination (**Fig. 4E, top**). Second derivative analysis of the ATR-FTIR spectra was performed to identify individual components under the intrinsically broad amide I band envelope (Barth and Zscherp, 2002). It revealed major contributions typical of β-sheet structures for apo-2H10, in agreement with CD data (**Fig. 4E, bottom**). The presence of 1633 and 1693 cm^{-1} bands simultaneously is most likely associated with an anti-parallel arrangement of β-strands in sdAb 2H10, which are typically characterized by a strong band at ~1630 cm^{-1} and a weaker one at ~1690 cm^{-1} (van de Weert et al., 2001;Barth and Zscherp, 2002). Upon heme ligation, there was a rearrangement in β structures with the β-turn band at 1668 cm^{-1} shifting to 1678 cm^{-1} and a lack of the contribution at 1693 cm^{-1}, which denotes a loss of the anti-parallel β-sheet fingerprint. In addition, a prominent feature arose at 1653 cm^{-1}, at a position in which both α-helices and disordered segments contribute and which cannot be further resolved at this stage. In summary, the combination of both CD and FTIR reveals that the sdAb 2H10 was composed of predominantly anti-parallel β-sheets, which undergo conformational changes upon heme binding.

We employed Resonance Raman (RR) spectroscopy to characterize further the heme moiety in the heme-2H10 sdAb complex. Upon excitation into the Soret electronic transition band, RR spectra display in the high frequency region (1300-1700cm^{-1}) core size marker bands (e.g. υ_4, υ_3, υ_2 and υ_{10}), which are sensitive to coordination, spin and redox state of the Fe-heme. A comparison of RR spectra of hemin versus the 2H10/heme complex (**Fig. 4F and 4G**) demonstrates that sdAb 2H10 binds to heme *b* in a specific manner. This was particularly evident from the changes in frequency and relative intensity of spin state sensitive υ_3, υ_2 and υ_{10} modes. Also, the bandwidth of the well defined υ_3 mode revealed a presence of one spin species (i.e. one type of ligation) in the complex. The υ_4 at 1377 and 1373 cm^{-1} in the heme-2H10 complex and heme, respectively, indicated a Fe^{3+} state of the heme Fe in both cases. The heme-2H10 complex revealed the υ_3 and υ_2 at 1508 and 1585 cm^{-1} respectively, characteristic for six coordinated low spin Fe-heme (**Fig. 4G**). These modes were found at 1492 and 1571 cm^{-1}, respectively in heme spectra, indicating a five-coordinated high spin (**Fig. 4F**) state, with most likely a Cl^- ion acting as the fifth axial ligand. The most probable candidates for heme axial ligands in the six coordinated low spin 2H10/heme complex were tyrosine and lysine besides cysteine, as suggested by the UV-Vis spectra. These aa are present as axial ligands in several hemoproteins as well as in the CDR1 of sdAb 2H10 (**Fig. 1F**) (Ferrer et al., 1993;Rosell et al., 1998;Li et al., 2011). Also, H_2O molecule, which in some heme proteins acts as a strong axial ligand, cannot be excluded as a potential 6th ligand. However, other amino-acids in the sdAb sequence cannot be excluded as candidates for coordinating heme Fe. Further experiments are required to disclose their nature, such as site-direct mutagenesis on those amino acids which were identified as the most likely candidates, and their subsequent characterization using for instance the previously described methods.

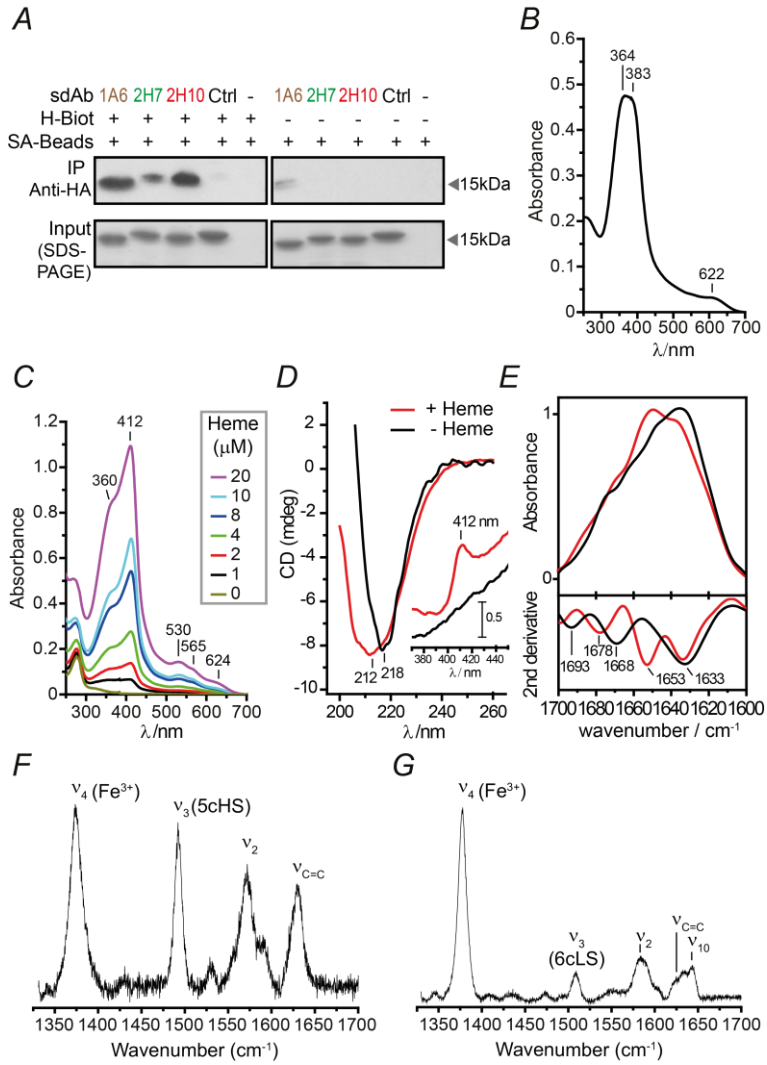

Figure 4. Characterization of sdAb binding to heme. (A) SdAbs bound to biotinylated heme in solution were pull- down using streptavidin (SA) beads and detected by western blot using anti-HA mAb. Input was measured by coomassie-based stain. (B) UV-Visible spectra of hemin. Soret region at approximately 363 nm and 383 nm and a CT band at 622 nm are shown. Data shown is representative of three independent experiments. (C) UV-Visible spectra of sdAb 2H10 incubated with increasing heme concentrations. Soret (412 nm), Q_1 (530 nm), Q_0 (565 nm) and CT (624 nm) bands are highlighted. Similar results were obtained for sdAb 1A6 and 2H7 (Suppl.Fig. 2A and 2B). (D) Far UV CD spectra of sdAb 2H10 in the apo (black) and heme-bound (red) forms. Shift from 212 to 218 is due to a heme-driven conformational rearrangement of the sdAb secondary structure. The inset shows the Soret region, with the appearance of 412 nm band, due to heme binding to the sdAb. (E) ATR FTIR absorption spectra (top) and second derivative (bottom) of sdAb 2H10 in the apo (black) and heme-bound (red) forms in the amide I region (1700-1610 cm^{-1}), showing structural modification upon heme coordination. High frequency RR spectra of (F) hemin and (G) sdAb 2H10 bound to hemin, obtained with 413 nm excitation.

3.4 Detection of cellular heme

We tested the sdAbs for their ability to detect intracellular heme *in situ* by immunofluorescence microscopy. Heme was readily detected in human HeLa cells using all three sdAbs, while background signal was obtained using a control sdAb, which does not recognize heme (**Fig. 5A**). Detection of cellular heme was confirmed by flow cytometry (**Fig. 5B**), and was fully inhibited when sdAb 2H10 was pre-incubated with increasing heme concentrations (**Fig. 5C**). Heme pre-incubation also reduced detection of cellular heme by sdAb 2H10 in immunofluorescence microscopy (**Fig. 5D**). Consistent with the *in vitro* data (**Fig. 2A**), pre-incubation of sdAb 2H10 with PP failed to inhibit cellular heme recognition, as assessed by flow cytometry (**Fig. 5E**). These data supports the notion that sdAb can be used to detect cellular heme, presumably "free heme". Further studies are required to fully understand whether the detected heme is “free”. Ideally, cells depleted of heme but with the capacity to incorporate exogenous heme should be used. Therefore, “free heme” would only be detected when provided exogenously. This would mimic the organism *Caenorhabditis elegans* that do not synthesize heme, acquiring it from exogenous sources (Sinclair and Hamza, 2015). However, such cells do not exist and the usages of drugs, such as succinylacetone for heme synthesis inhibition might not to be sufficient to reduce significantly intracellular heme and prolonged treatment with this drug leads to increased cell death.

Furthermore, we cannot exclude that the heme detected by the sdAb might be released from intracellular hemoproteins that were denaturated during cell treatment for immunoFluorescence/Flow Cytometry assay. Thus the methanol/paraformaldehyde and eventually triton X-100 used for cell fixation and permeabilization, respectively, will induce protein denaturation. These treatments might also promote some conformational changes in the hemoproteins which might expose their heme groups that become accessible to the sdAb. To address these questions, further studies are required.

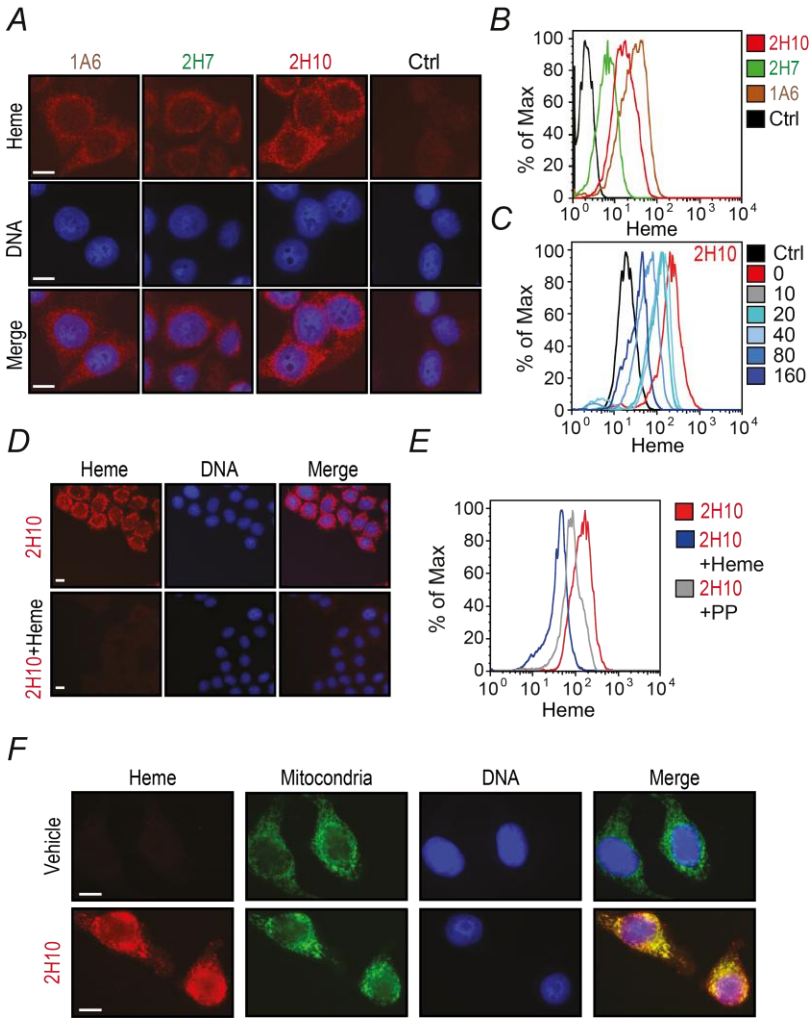

Figure 5. Detection of intracellular heme by sdAbs. (A) Intracellular heme (red) and DNA (blue) were detected by immunoflurescence in HeLa cells using anti-heme sdAbs 1A6, 2H7 and 2H10 and DAPI, respectively. Staining with a control sdAb (Ctr.) that does not recognize heme is also shown. Images are representative of three independent experiments. Scale bar, 10 µM. (B) Intracellular heme detection by flow cytometry in HeLa cells, using the same sdAbs as in (A). Histograms are representative of three independent experiments. (C) Same as (B) with pre-incubation of sdAb 2H10 with increasing concentrations of hemin (0-160 µM) in solution. Data is representative of three independent experiments. (D) Same as (A) using sdAb 2H10 pre-incubated or not with hemin (160 µM) in solution. Data is representative of three independent experiments. (E) Same as (C) with sdAb 2H10 pre-incubated with heme (160 µM) or PP (160 µM). Data is representative of three independent experiments. (F) Same as (A) in HeLa cells co-stained with MitoTracker® for mitochondria localization (Green). Heme was detected by the sdAb 2H10 (red) and DNA with DAPI (blue). Merged images show co-localization of heme with mitochondria (yellow). All images with a scale bar, 10 µM.

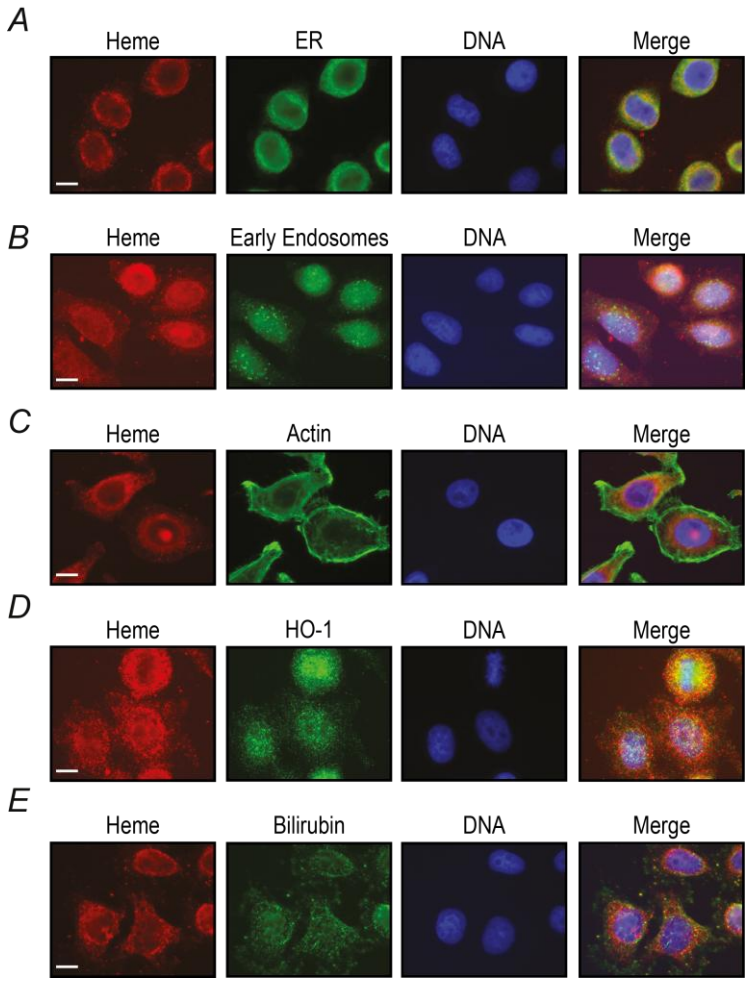

Figure 6: **Detection of heme in different cellular organelles.** Heme (red) and DNA (blue) detected by immunoflurescence in HeLa cells using the sdAb 2H10 and DAPI, respectively. Cells were co-stained (Green) with (A) anti-Calnexin for endoplasmatic reticulum (ER) (B) anti-EEA-1 for endosomes, (C) a toxin phalloidin–tetramethylrhodamine B isothiocyanate phalloidin for F-actin, (D) anti-bilirubin mAb and (E) anti-HO-1 polyclonal Ab. Merged figures show co-localizations (yellow). Data shown is representative of three independent experiments. All images with a scale bar, 10 µM.

Considering that sdAb are detecting cellular “free heme”, we asked whether "free heme" can be detected in different intracellular compartments, HeLa cells were co-stained for heme and for specific cellular compartments, including nucleus (DNA/DAPI), mitochondria (MitoTracker), endoplasmic reticulum (calnexin), early endosomes (EEA1) and cytoskeleton (F-actin)(**Fig. 5F and Fig. 6**). The majority of

sdAb 2H10 signal, corresponding to the presence of "free heme", overlapped with the mitochondria (**Fig. 5F**), the main organelle for heme biogenesis and a minor portion overlapped with the ER and endosomes (**Fig. 6A and 6B**). In addition, sdAb 2H10 signal was also co-localized with the heme-degrading enzyme HO-1 and to a lesser extent with bilirubin (**Fig. 6D and 6E**). These observations reflect most probably the relative affinity of sdAb 2H10 to “free heme” (~10^{-7}M; **Fig. 1F**) plausibly revealing the presence of a major cellular heme pool within the mitochondria as well as associated with HO-1, outside the mitochondria.

The different patterns observed in the cellular heme detection by the sdAb (**Fig, 6 A-E)** are in agreement with inherent variations on the assays as those were performed in different days and consequently with different cells and the images between the different assays were not normalized.

3.5 Quantitative analyzes of circulating heme after acute hemolysis in vivo.

Having characterized the heme binding properties of sdAb, we set to test whether these sdAbs can be used to monitor, in a quantitative manner, heme accumulation in the plasma during hemolytic conditions. To address this, we used a well-established experimental model in which phenylhydrazine (PHZ) administration induces acute hemolysis in mice (Itano et al., 1975). Under steady state conditions, the mean plasma concentration of heme in adult C57BL/6 mice was 23,4 µM, as assessed using a formic acid based assay and consistent with similar values obtained using different assays (Vinchi et al., 2013). Heme concentration in plasma increased to an average of 95.6 µM, as assessed 3 hours after PHZ administration, a value maintained for 12 hours thereafter, the last time point analyzed (**Fig. 7A**). To further characterize heme concentration in plasma, we developed a sandwich ELISA in which heme is captured by solid phase bound sdAb 1A6 and detected by sdAb 2H7 (**Suppl. Fig. 4**). Given the affinity of sdAb 1A6 towards heme, *i.e.* 10^{-7} M, this assay detects heme only if not bound to other plasma proteins or ligands with an affinity higher than 10^{-7} M, *i.e.* “free heme”. Under steady state conditions “free heme” in plasma remained undetectable, <0.15 µM (**Suppl. Fig. 4**) and induction of acute hemolysis by PHZ did not result in detectable accumulation of “free heme” in

plasma, which confirms the physiological capacity of plasma heme-binding proteins to scavenge heme, with an affinity higher than 10^{-7} M.

We then asked whether the accumulation of heme in plasma associated with acute hemolysis increases heme bioavailable for cellular uptake (White et al., 2013). Under steady state conditions, cellular heme uptake was negligible, as assessed by HRP reporter activity in HEK293 cells exposed to plasma derived from PHZ-untreated animals (**Fig. 7B**). Hemolysis was associated with increased heme bioavailability, as revealed 3 to 12 hour after PHZ administration (**Fig. 7B**). Only a small fraction, ~ 4 µM corresponding to 4% of the total heme detected in plasma, became bioavailable for cellular uptake, plausibly reflecting the high buffering capacity of plasma heme scavenging proteins.

To monitor heme buffering capacity (HBC) in plasma we reemployed a similar sandwich ELISA with one major modification: the plasma was first pre-incubated with increasing amounts of heme prior to the assay, so as to measure the concentration of "free heme" at which plasma reach half-saturation of its total HBC. Plasma HBC in adult C57BL/6 mice at steady state was in average ~23.8 mM (**Fig. 7C**), exceeding by ~100 times the total amount of heme detected in plasma (**Fig. 7A**). Acute hemolysis was associated with a decrease of plasma HBC, to an average 16,8 mM, as assessed 6 hours after PHZ treatment, returning to steady state levels 12 h later (**Fig. 7C**). This provides further confirmation that although acute hemolysis is clearly associated with the accumulation of heme in plasma, plasma does have an extremely high buffering capacity, which presumably safeguards against the deleterious effect of "free heme". This higher HBC most probably is provided by proteins or other molecules in the plasma with capacity to binding heme, such as albumin, immunoglobulins, hemopexin and lipoproteins (Camejo et al., 1998;Paoli et al., 1999;Wardell et al., 2002;Dimitrov et al., 2007;Larsen et al., 2012;Hadzhieva et al., 2015).

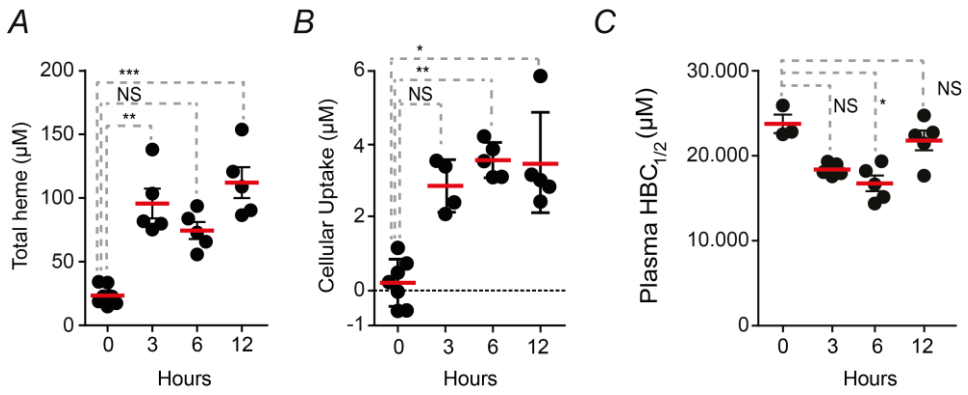

Figure 7. Heme accumulation in plasma during acute hemolysis in mice. (A) Heme concentration in plasma of control mice (0) and mice receiving PHZ. (B) Cellular heme uptake from the plasma of control mice (0) and mice receiving PHZ, monitored by the HRP-reporter HEK 293 cells. (C) Heme binding capacity (HBC) of plasma from control mice (0) and in mice receiving PHZ. *p < 0.05, ** p < 0.01, ***p < 0.001. Each circle corresponds to an individual mouse. Red bars represent mean ± STD,

3.6 Modulation of heme biologic activity by sdAb

We next asked whether heme binding by sdAb 2H10 interferes with the redox activity of heme, as assessed by the modulation of electron exchange between heme and ascorbic acid in the presence of H_2O_2. As expected, heme catalyzed ascorbic acid oxidation in a dose-dependent manner, that is, higher heme concentrations (0-5 µm) increased the extent of ascorbate depletion, as detected by HPLC (**Fig. 8A and 8B**). This pro-oxidant effect of heme was significantly attenuated in the presence of sdAb 2H10, as compared to equimolar concentrations of control sdAb or human serum albumin (**Fig. 8A**). Importantly, sdAb 2H10 also inhibited the pro-oxidant activity of heme/H_2O_2 in a concentration-dependent manner that is, higher sdAb concentrations reduced ascorbate depletion, as detected by HPLC (**Fig. 8B**). At each concentration tested, the sdAb 2H10 was more effective than the control sdAb or human serum albumin in terms of preventing ascorbate depletion (**Fig. 8B**).

To determine whether heme binding to sdAb 2H10 prevents cellular heme uptake, we used a horseradish peroxidase (HRP)-based heme reporter assay previously shown to reflect intracellular heme availability in mammalian cells (White

et al., 2013). HEK293 cells were treated with succinylacetone, an inhibitor of heme synthesis to prevent " *de novo*" synthesize of heme and consequently its incorporation into apoHRP and restored its activity, As expected, treated cell showed greatly reduced levels of HRP activity, while supplementation with either exogenous heme (0.5µM) or sdAb 2H10 bound to heme fully restored HRP activity (**Fig. 8C**). This was not recapitulated when cells were provided with heme bound to the heme scavenger hemopexin (HPX)(**Fig. 6C**). Consistent with this observation, heme-2H10 complexes sensitized primary mouse hepatocytes to undergo programmed cell death in response to tumor necrosis factor (TNF) in a similar manner as heme alone(Larsen et al., 2010;Gozzelino and Soares, 2011) (**Fig. 8D**). This suggests that heme binding by sdAb 2H10 does not inhibit heme intracellular access and hence its cytotoxic effect (Larsen et al., 2010;Gozzelino and Soares, 2011).

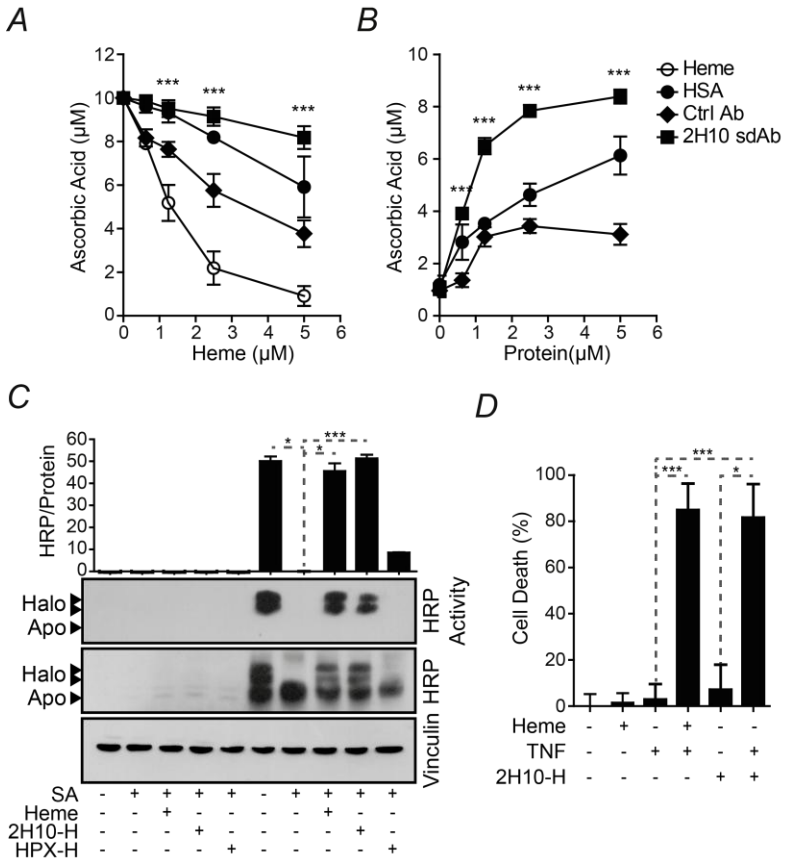

Figure 8. Targeting heme biologic activity with sdAbs. Ascorbate oxidation by heme in the presence or absence of the sdAb 2H10, Ctrl sdAb or human serum albumin (HSA). (A) Increasing heme concentrations. (B) Increasing protein concentrations. Results shown are the mean ± SEM from three (B) and four (A) independent experiments. (C) Cellular heme content in HRP reporter HEK 293 cells treated (+) or not (-) with SA and exposed (+) or not (-) to "free heme", heme bound to sdAb 2H10 (2H10-H) or heme bound to HPX (HPX-H). Results shown are the mean ± STD from three replicates in one experiment representative of three independent experiments. (D) Viability of primary mouse hepatocytes exposed (+) or not (-) to "free heme" (5µM), 2H10-H and exposed or not TNF (5ng/mL) as indicated. Results shown are the mean ± SD from six samples in one experiment representative of three independent experiments. *$p < 0.05$, ***$p < 0.001$ Asterisks (***) indicate that the mean value of each point of sdAb 2H10 differs from the corresponding mean point value of Ctrl sdAb at the $p < 0.001$.

4. DISCUSSION

75-80% of bioavailable Fe in mammals is contained inside heme, whereas the remaining 20-25% is present in the form of non-heme Fe (Gozzelino and Soares, 2014). Heme is found essentially within hemoproteins and specifically in Hb contained inside red blood cells (RBC) (Schneider et al., 2007). Several pathologic conditions are incompatible with the maintenance of RBC integrity thus leading to release of Hb in plasma, i.e. hemolysis. Because of the sheer number of RBC (~2-3x10^{13} in adult humans) as well as their high Hb (~3x10^{8} molecules/RBC) and heme (~1.2x10^{9} heme per RBC) (Smith, 1980), undetectable levels of hemolysis clinically can be associated with release of significant amounts of biologically active Hb. Upon release from RBC, tetramers of cell-free Hb dissociate into dimers that are readily oxidized, releasing their non-covalently bound heme *b* (Gozzelino et al., 2010). Several lines of evidence suggest that once released from Hb, heme *b* can act in a cytotoxic (Seixas et al., 2009;Gozzelino et al., 2010;Larsen et al., 2010), vasoactive and pro-inflammatory (Graca-Souza et al., 2002;Figueiredo et al., 2007) manner, promoting the pathologic effects of hemolysis. This appears to be true for pathologic conditions associated with varying levels of hemolysis, such as, malaria (Pamplona et al., 2007;Ferreira et al., 2011), severe sepsis (Larsen et al., 2010) or sickle cell disease (Ghosh et al., 2013;Vinchi et al., 2013;Belcher et al., 2014). It becomes apparent that mechanisms controlling heme homeostasis are likely to regulate the pathogenesis of these and probably other related diseases (Schaer et al., 2013).

From the context of these pathophysiologic conditions, there are currently no analytical methods available that provide quantitative and qualitative information on the pathologic effect of heme, as it accumulates in plasma when released from cell-free Hb. Here we describe a panel of sdAbs that detect specifically heme upon its release from hemoproteins (**Fig. 1, 2 and 3**). The affinity of these sdAbs is in the range of 10^{-7} M (**Fig. 1F**), such that the sdAbs detect only heme which is not bound to scavenging systems with an affinity higher than 10^{-7} M. This makes these sdAbs specific for what we refer to as “free heme”, *i.e.* redox active heme that is loosely bound to proteins or to other biologic compounds with an affinity lower than 10^{-7} M.

We identified three sdAbs, *i.e.* 1A6, 2H7 and 2H10 that recognize heme, of which sdAb 2H10 appears to be the most specific (**Fig. 1, 2 and 3**). The sdAb 2H10 does not recognize other closely related molecular structures, such as the tetrapyrroles biliverdin, bilirubin, metal-depleted or Zn PP (**Fig. 2 and 3**). Moreover, sdAb 2H10 does not recognize heme when contained within several tested hemoproteins or bound to human serum albumin (**Fig. 3C**). Structural properties of the 2H10/heme complex reveal that this sdAb interacts specifically with heme, presumably *via* a cysteine as the fifth ligand and a lysine or tyrosine as the sixth axial ligand (**Fig. 4C to 4G**) (Li et al., 2011). We cannot exclude that others ligands, such as water or aa from sdAb sequence act as a sixth ligand of heme Fe. From a biological perspective, immunofluorescence microscopy revealed that sdAb 2H10 detects the "free heme" in human cells, mainly in the mitochondria while a minor portion was also detected in the secretory pathway (**Fig. 5F**). This is consistent with the notion that endogenous cellular heme is synthesized in the mitochondria while extracellular heme is obtained *via* endocytic pathways (White et al., 2013). Furthermore, sdAb 2H10 also detected "free heme" in close vicinity to the heme-degrading enzyme HO-1. We can speculate that this sdAb is detecting the substrate of HO-1 in cells, and that heme bound to ligands with affinity higher than 10^{-7} M is probably not a substrate for HO-1 (**Fig. 6D**). Cellular "free heme" was also, although less frequently, detected in the vicinity of bilirubin, an end product of heme catabolism no longer associated with HO-1 (**Fig. 6E**). This suggests that "free heme" can be found in the vicinity of heme catabolizing events, where substrate, enzyme and end-product are in the same region. While consistent with the notion that sdAb 2H10 recognizes specifically cellular "free heme", we cannot exclude that this sdAb may also react with heme when loosely or transiently bound with low affinity ($<10^{-7}$ M) to cellular proteins or lipids. We also need to consider that the sdAb might be detecting heme that was released from denaturated hemoproteins during cell treatment for immunoFluorescence/Flow Cytometry assay. Besides that, this type of treatment could also induce conformational changes in the hemoproteins, making their heme groups accessible to the sdAb. Nevertheless, further studies are required to elucidate it.

The development of methodologies allowing for the quantification and eventually the specific targeting of “free heme” as it accumulates in plasma during hemolysis, should provide further information not only about the participation of “free heme” in the pathogenesis of hemolytic conditions, but presumably also allow for its specific therapeutic targeting.

Using a newly developed sdAb-based sandwich ELISA (**Suppl. Fig. 4**) we found that despite heme accumulation during acute hemolysis, there is very little or no “free heme” in plasma; the assay has a linear range between 0.15-2.5 µM (**Suppl. Fig. 4**). This result is in contradiction with previous results that described an increase in “free heme” associated with the pathology of hemolytic diseases (Pamplona et al., 2007;Ferreira et al., 2008;Seixas et al., 2009;Larsen et al., 2010;Gozzelino et al., 2012). The discrepancies between those studies seem to be correlated with the methodologies used to detect “free heme”. In one of those studies, “free heme” is defined as protein-free heme and was determined after removing the proteins from plasma by filtration to retain all protein with a molecular weight higher than 3kDa (Pamplona et al., 2007;Seixas et al., 2009). The protein-depleted plasma was then used for heme quantification using a chromogenic assay from a QuantiChrom heme assay kit (Biosystems). Although the major plasma proteins are removed by this method, it does not allow to assess whether heme is “free” as it still might be associated with other molecules of lower molecular weight than 3kDa. The authors of those papers did not determine whether some proteins were still present in the filtered plasma, thus excluding this possibility. While in another study, “free heme” in plasma is defined as non-hemoglobin bound heme and was determined by subtracting the value of total heme, determined by a peroxidase assay, from total hemoglobin concentration, determined through UV-visible spectra in which purified hemoglobin was used as standard (Larsen et al., 2010;Gozzelino et al., 2012;Jeney et al., 2014). This method has several limitations that can compromise the precision of “free heme” quantification; i) peroxidase activity is not directly proportional to the heme concentration, since the peroxidase activity of heme is dependent of the protein to which it is bound as well as to the solvent, as exemplified for heme vs cytochrome c (Laszlo and Compton, 2002),

albumin or hemopexin (Grinberg et al., 1999); ii) the UV-visible spectra of the different hemoproteins are very similar, displaying the typical Soret and Q bands and sometimes CT band, and the absorption peaks vary with for instance the solvents, heme ligands and hemoproteins oxidation state (Brill and Sandberg, 1968;Kuzelova et al., 1997;Zijlstra and Buursma, 1997;Mauk et al., 2007;Nicoletti et al., 2008). Considering that in plasma there are several hemoproteins, including hemoglobin (mainly upon hemolysis) the UV-visible obtained from plasma will not be specific for hemoglobin (Zijlstra et al., 1991;Zijlstra and Buursma, 1997) and other hemoproteins will interfere. All together, these results might lead to misinterpretation of the values obtained for "free heme" quantification. For that reason, this project was developed aiming to overcome those limitation and better understand the role of "free heme" in the pathology of immune mediated inflammatory diseases.

Nonetheless, acute hemolysis is associated with increased of total heme and heme bioavailability reflected by cellular heme uptake as well as reduced plasma HBC, as revealed using another sdAb based sandwich ELISA (**Fig. 7**). This confirms that acute hemolysis is associated with heme accumulation in plasma, and consequently with an increase of reactive heme available for cellular uptake. Despite a transient decrease in plasma HBC during acute hemolysis, heme is probably always bound to plasma compound(s) with an affinity $>10^{-7}$ M, hence not detectable by the sdAbs based ELISA. The observation that plasma HBC decreases only transiently, upon which it rapidly returns to steady state (**Fig. 7**), argues for a tight regulatory mechanisms restoring systemic heme homeostasis. This mechanism is probably not mediated by HPX, given that deletion of the HPX gene has no effect on the overall plasma HBC in mice (data not shown).

When bound to soluble heme, sdAb 2H10 inhibited its pro-oxidant activity (**Fig. 8A and 8B**), as assessed by ascorbate oxidation, used hereby as a proxy of heme redox activity. While plasma heme scavengers such as albumin also diminish heme pro-oxidant activity, sdAb 2H10 is more efficient in that respect (**Fig. 8A and 8B**). This may be particularly useful when envisaging the use of anti-heme sdAb as a therapeutic approach to target the pro-oxidant effects of extracellular soluble "free

heme" and prevent its pathogenic effects in the context of immune-mediated inflammatory diseases. The observation that the described sdAb do not target heme when contained in Hb, Mb or cytochromes should make such an approach far less toxic, as compared to other approaches affecting heme stability in these hemoproteins (Pamplona et al., 2007;Gozzelino et al., 2010). The use of albumin to target heme could also be considered, however little is known about its role in prevention of the cytotoxic effect of heme. For instance, it was demonstrated that albumin can also bind other molecules that modulate its heme binding, such as fatty acids (Fasano et al., 2005;Ascenzi and Fasano, 2009), which can compromise the efficiency of albumin to prevent the cytotoxic effect of heme.

In conclusion our present study reveals that anti-heme sdAbs can be used to detect and quantify "free heme" as well as to modulate its biologic activity. Further applications of such sdAbs shall be determined as these become available to the community.

5. EXPERIMENTAL PROCEDURES

Tetrapyrroles preparation - Concentration of different tetrapyrroles (Frontiers Scientific®, Utah, USA) in solution was determined spectrophotometrically using appropriated solvents. Briefly, heme (i.e. hemin) stock solutions were prepared in 0.1 M NaOH, buffered with 0.1 M HCl to pH 7.4. Alternatively, hemin was diluted, as for all other tetrapyrroles, in dimethyl sulfoxide (DMSO). Hemin concentration was determined at λ_{405nm} using a extinction coefficient (E_{mM}) of 85,82, DeutP (λ_{392nm}; E_{mM}=170) and GaPP (λ_{413nm}, E_{mM}=249) (43). PP (λ_{408nm}; E_{mM}=297) concentration was determined in 1.5 M HCl (43), ZnPP (λ_{415nm}; E_{mM}=150) in ethanol (43); biliverdin (λ_{377nm}; E_{mM}=51,5) in methanol (44) and bilirubin (λ_{451nm}, E_{mM}=60) in chloroform (45). CoPP, SnPP and FePPCH3 concentrations were calculated gravimetrically according to their molecular weight (MW) =654.6, 750.26 and 679.99, respectively.

Heme biotinylation – The procedure used was modified from the original protocol (46). Briefly, hemin in dimethylformamide (DMF; 4.4 mg/mL) was incubated with 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate methanaminium (HATU) and N,N-diisopropylethylamine (30-60 min.; RT). N-[-5-(Hydrazinocarboxy) pentyl]-D-biotinamide (biotin) in dimethylsulfoxide (DMSO) was added and after 60 min. at RT the reaction mixture was applied onto a C18 reverse-phase analytic high performance liquid chromatography (HPLC) column (COSMOSIL 5C18-ARII, Nacalai Tesque) to separate biotinylated heme from “free heme” and biotin. Fractions were dryed in a SpeedVac (SpeedVac Plus SC110A attach to Vaccum System Plus UV400A) at <15ºC.

(MALDI)-Time-of-flight mass spectrometry (TOFMS) Analysis – Hemin and biotin in 0.2M NaOH were spotted directly onto the MALDI target plate and mixed 1:1 with 2,5-dihydroxybenzoic acid (DHB; 10 mg/mL) in 50% v/v acetonitrile (ACN) with 5% v/v formic acid and air dried. Heme-biotin in 50% (v/v) ACN was spotted directly onto a MALDI plate using the Maldi matrix described above. Data was acquired in positive reflector MS mode in a mass spectrometer (4800 plus MALDI-

TOF/TOF; AB Sciex) and collected (4000 Series Explorer Software v.3.5.3; Applied Biosystems). Mass spectrometer calibration was performed using angiotensin II (1046.542 Da), angiotensin I (1296.685 Da), Neurotensin (1672.918 Da), Adrenocorticotropic hormone (ACTH)(1–17)(2093.087 Da) and ACTH (18–39)(2465.199 Da)(Peptide Calibration Mixture 1, LaserBio Labs). Each reflector MS spectrum was collected using 500 laser shots per spectra and a fixed laser intensity of 3300 V.

Phage library and selection (47) – To select sdAb´s against heme we used a synthetic VL sdAb phage display library previously tested successfully against several targets (Goncalves et al, submitted). The library repertoire ($8x10^9$ independent clones) was generated on a highly soluble and stable VL scaffold by randomization and accommodating a maximum of 26 aa in CDR1 and 22 aa in CDR3 (25). The selection process was divided into four main steps: (i) incubation of phage-sdAb repertoire with heme-biotin; (ii) streptavidin capture and washing to remove non-specific phages-sdAb; (iii) heme competition for phage-sdAb elution and (iv) re-amplification of antigen-specific phages-sdAb. Five rounds of binding, elution and amplification were performed to generate sdAbs with high binding activities and specificity. Briefly, heme-biotin (1µg/µL) was pre-incubated (2h; RT) with the sdAb VL phage library (~10^{10}/mL). The selection procedure consisted in using streptavidin coated magnetic beads (Dynabeads; M280) to capture under a magnetic field those phages bound to the heme-biotin. Unbound or low affinity bound phages were washed out (PBS 0.5% tween 20, 5x). Bound-phages were released from the heme-biotin with a competition step by addition of excess hemin (100µM in PBS; 1h; RT; 2x). The recovered phages were used to infect *E. coli* ER2738 (phage display optimized strain; λ_{600nm}=0.6; Biocat; 4mL; 15-30 min. 37ºC). Phage-infected ER2738 bacteria were grown in Super Broth (10µg/mL tetracycline, 100µg/mL ampicillin) and plated in Luria Broth (LB) agar (100µg/mL ampicillin) at 37ºC. Single clones were randomly selected and screened by PCR to insure the presence of VL sequences. Bacteria growing in liquid medium were then infected with VCSM13 helper phages (10^{12}-10^{13} pfu in 4 mL; 15-30 min.; 37ºC), re-selected

for kanamycin (100µg/mL) resistance and left overnight at 37ºC for phage production. After 16h, the selected phages were precipitated using PEG-8000 (4% w/v), NaCl (3% w/v)(1 h on ice), centrifuged (8000g; 30 min. 4ºC), re-suspended (PBS, 15% Glycerol, 1 h in ice), centrifuged (16000g, 10-20 min. 4ºC) and filtered (0.22µm). The resulting "purified phages" were used for one additional panning with ten washing cycles (PBS 0.5% Tween 20) and three more additional pannings with fifteen washing cycles (PBS 0.5% Tween 20). *E. coli* (TOP10F' strain; λ_{600nm}=0.6) were infected with phages selected from the fourth panning (4mL; 15-30 min. 37ºC), grown (Super Broth, 10µg/mL tetracycline and 100µg/mL ampicillin), plated (LB agar, 100µg/mL ampicillin) and the presence of VL sequences was confirmed again by PCR. Phage-infected bacteria were grown (Super Broth, 10µg/mL tetracycline and 100µg/mL ampicillin, ON), pcomb3x phagemid DNA was extracted (Midi prep. Quiagen), isolated and digested with SfiI (Fermentas) for the extraction of DNA encoding sdAb fragments. These were purified from agarose electrophoresis, cloned into a modified PpT7-FLAG vector (Sigma)(Technophage SA, Portugal and used to transform *E. coli* (BL21 (DE3), Promega). Bacterial clones were grown in auto induction medium (Novagen), lyzed (PBS, 20% Bugbuster, Novagen, ON, 4ºC) and protein extracts containing the sdAb fragments were obtained (16000g, 15 min., 4ºC) for selection according to heme binding capacity.

SdAb expression and purification – Twenty His-HA-tagged sdAbs were selected according to heme binding capacity and ten of those were expressed and purified, based on a modified protocol (Goncalves et al, manuscript submitted). Briefly, the sdAbs cloned in a pT7 expression vector were transformed in *E. coli* BL21 (DE3) cells and sequences were confirmed (Macrogen DNA Sequencing service), before expression and further purification. For protein expression, one liter of LB, containing 50 µg/mL carbenicillin was inoculated with 10 mL of overnight culture of bacterial cells transformed with pT7-VL´s plasmids and grown to exponential phase (λ_{600nm}=0.6-0.9) at 37ºC. Expression was induced by the addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and growth during 6 h at 37ºC. Cells were harvested by centrifugation (4000 g for 15 min at 4 ºC) and resuspended in 50 mL

equilibration buffer (50 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 10 mM imidazole, pH 7.8) supplemented with protease inhibitors (Roche).

Cells were lysed by sonication (20min., ice) and the inclusion bodies containing the sdAbs were recovered by centrifugation (12096g, 30 min., 4ºC). Then, the pellet was washed (50mM HEPES, 1M NaCl,10mM imidazole, 5mM $CaCl_2$, 2M urea, 1mM β-mercaptoethanol (β-ME) pH 7.8), sonicated (20 min., ice) and collected by centrifugation (12096g, 30 min., 4ºC). The inclusion bodies were then re-suspended in a 6 M Urea buffer containing benzonase (>250U, Sigma)(50mM HEPES, 1M NaCl, 10mM Imidazole, 5mM $CaCl_2$, 6M urea, 1mM β-ME, pH 8.0) and incubated overnight (4ºC, under agitation) for protein denaturation. Next day, the denatured sdAbs were purified under denaturing conditions using a His Trap Fast Flow (FF) column (GE Healthcare), and washed twice with 20 and 30 mM of imidazole to remove contaminants (i.e. non specific proteins)(50 mM HEPES, 1M NaCl, 6M Urea, 1mM β-ME, 5mM $CaCl_2$, and 20 or 30 mM Imidazole pH 7.8). The sdAb were eluted in high concentrated imidazole buffer (50 mM HEPES, 1M NaCl, 500 mM Imidazole, 6M urea, 1mM β-ME, 5mM $CaCl_2$, pH 7.8). Re-folding was done using a step-wise dialysis protocol on previous described protocols (30). Briefly, purified sdAb was initial dialyzed to Tris buffer containing 10 mM β-ME to maintain the reduction state of protein and 6M Urea protein (50 mM Tris-HCl, 1mM EDTA and 200 mM NaCl)(24h, 4ºC). The 10mM β-ME was then removed by dialysis using the same buffer without β-ME, to promote thiol groups oxidation and consequently disulfide bond formation (48h, 4ºC). The protein was then refolded by sequential dialysis against 3, 2, 1, 0.5 and 0 M urea with or without additives (24h, 4ºC, each dialysis step). In the buffers containing 1 and 0.5 M of urea 0.4 mM oxidized (GSSG) and 4mM of reduced (GSH) glutathione and 400mM L-arginine were add. These additives were proven to help in the protein refolding (30). The refolded proteins were then centrifuged to remove protein precipitates (4000g, 4ºC, 10 min.), aliquoted and stored (-80ºC) until used. The purity of the sdAbs was assessed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with

15% acrylamide composition under denaturating conditions followed by based coomassie stain (Instant Blue, Gentaur, 30 min., RT) and by Western blotting.

SdAb biotinylation – It was performed using EZ-Link NHS-PEG4-Biotinylation kit (Pierce). Briefly, 20-fold molar excess of biotin was added to 1.1 mg/mL of sdAb 2H7 (in PBS) and incubated (ice, 2h). Free biotin and protein were removed using a desalting column (Thermo Zeba Spin Desalting column, Pierce)(1000g, 2 min.) pre-equilibrated in PBS. The biotinylated-sdAb was stored at -80ºC until used. Biotin incorporation was confirmed using 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay. Briefly, biotinylated-sdAb was mixed (1/10) with a solution of HABA/Avidin (0.5 mg/mL avidin, 0.3 mM HABA, PBS) in a 96 well plate and λ_{500nm} was measured after and before addition of biotinylated protein using a microplate reader (Vitor 3 luminometer; Perkin Elmer).

Western Blotting – Proteins were subjected to 15% SDS-PAGE gel electrophoresis (15 V, 60 min.), transferred into methanol activated polyvinyldifluoridine membrane (2 min.), washed (3x in H_2O and 3x T-TBS; 20 mM Tris pH 7.5, 150 mM, 0.05% Tween-20) and blocked (5%-skim milk in T-TBS; 1h-overnight; RT). sdAbs were detected with a horseradish peroxidase (HRP) conjugated rat anti-HA monoclonal Ab (mAb)(1/2000, Roche ®). Peroxidase activity was revealed using SuperSignal West Pico Chemiluminescent substrate (Pierce) in a photoradiograph (Kodak Biomax Light Film; Eastman Kodak). ExPASy - ProtParam tool was used to estimate the MW and isoelectronic point of sdAbs.

ELISAs – To screen sdAb for heme recognition, 96 well ELISA plates (Costar 3690) were coated with hemin (100µM) in carbonate buffer (0.1M $NaHCO_3$, pH 8.6)(1h, RT or overnight at 4ºC), washed (3x, PBS, 0.1% Tween 20) and blocked in protein free blocking buffer (Pierce; 1h, RT). Plates were washed (4x, PBS, 0.1% Tween 20) and purified sdAbs diluted in PBS were added (1h 30 min., RT). Plates were washed (5x PBS, 0.1% Tween 20) and heme-bound sdAbs were detected using a rat anti-HA mAb (3F10; Roche; 0.1µg/mL in protein-free blocking buffer).

Plates were washed (5x PBS, 0.1% Tween 20) and the primary mAb was detected using an alkaline phosphatase labeled rabbit anti-whole rat IgG (Sigma) polyclonal Ab (1 hour, RT) diluted (1/2000 dilution) in protein free blocking buffer (Pierce). Alkaline phosphatase was revealed with para-Nitrophenylphosphate (pNPP, 1mg/mL; Sigma). To evaluate the total amount of protein, sdAb were coated in 50 mM carbonate/bicarbonate, pH 9.6 (1h; RT or 16; 4ºC) in 96 well ELISA plates (Costar 3690) and the rest of the procedure was performed as described above. Absorbance was measured in a microplate reader at λ_{405nm} (Vitor 3 luminometer; Perkins Elmer).

Sandwich ELISA was developed to measure "free heme" – SdAb 1A6 (5 µg/mL in 50 mM carbonate/bicarbonate, pH 9.6) was used to coat (overnight 4ºC) 96 well ELISA plates (Costar 3690). Plates were washed (5x 0.1% Tween 20 PBS) and blocked (2h, RT) using protein free blocking buffer (Pierce). Hemin (0.15-2.5 µM; used as standard) or plasma diluted in PBS were added (1h30, RT) and plates were washed (5x 0.1% Tween 20 PBS). The captured "free heme" was detected using biotinylated sdAb 2H7 (5 ng/µL in PBS), plates were washed (5x 0.1% Tween 20 PBS) and the biotinylated sdAb was detected using Alkaline phosphatase conjugated ExtrAvidin (1/2500 dilution, Sigma) in protein blocking buffer. Plates were washed (5x 0.1% Tween 20 PBS) and Alkaline phosphatase was revealed using para-Nitrophenylphosphate (1mg/mL; Sigma). Absorbance was measured in a microplate reader at λ_{405nm} (Vitor 3 luminometer; Perkins Elmer).

Heme binding capacity – Heme binding capacity was determined based on the same sandwich ELISA as described above. Plasma diluted in PBS was pre-incubated (1h30, RT, agitation) with increasing concentration of hemin and added to the plates.

Heme competition assays – SdAbs (2.5 µg/mL) were pre-incubated (90 min., RT, mild agitation) with different tetrapyrroles or hemoproteins and ELISA was

performed as described above. Concentrations of tetrapyrroles or hemoproteins used for pre-incubation are expressed as heme molar equivalents.

Biacore – The affinities of sdAbs towards heme were obtained using surface plasmon resonance (SPR)(BIAcore 2000, BIAcore Inc.). Briefly, sdAbs were captured on a CM5 chip using amine coupling at ~800 resonance units (RU). Heme at 0-3000 nM was injected for 4 min and sdAb-bound heme was allowed to dissociate (10 min.) before matrix regeneration (10 mM Glycine, pH 2.5). The signal was subtracted from that of an immobilized cell to generate sensorgrams of the amount of bound heme as a function of time. The running buffer, HBS-P (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20; BIAcore) was used for all sample dilutions. BIAcore kinetic evaluation software (version 3.1) was used to determine dissociation constant (KD) from the association and dissociation rates using a one-to-one binding model. An irrelevant sdAb was used as a negative control.

SdAb pull-down assay – SdAb binding to soluble hemin was assessed by a pull-down assay. Briefly, sdAbs were incubated (5h, 4ºC, agitation) with or without heme-biotin and half of the reaction mixture was incubated (2h, RT, agitation) with of Streptavidin-Dynabeads (M-280 Streptavidin, Invitrogen), previously blocked (5h, RT) with protein free blocking buffer (Pierce). Mixture was washed (3x; 5 min.; 0.05 Tween 20 in PBS) and magnetic beads were captured (5 min. magnetic field) according to manufacture instructions. SdAbs-heme-biotin complexes were collected (1 min., magnetic field) and incubated (20 min., 100ºC, agitation) in loading buffer (50mM Tris-HCl pH 6.8, 2%SDS, 10% Glycerol, 1% β-ME, 12,5 mM EDTA, 0.02% Bromophenol blue)(pull-down). The other half of the reaction mixture was denaturated (100ºC, 10 min.) in loading buffer. Proteins were applied into a 15% SDS-PAGE gel under denaturating conditions and stained with a coomassie-based stain (Instant Blue, Gentaur, 30 min., RT). Alternatively, sdAb were detected by Western blotting using a rat anti-HA horseradish peroxidase (HRP) conjugated mAb (1/5000, Roche ®)(1h, RT).

Spectroscopy assays – UV-Visible spectra were recorded on a PerkinElmer Lambda 25, UV/Vis spectrometer, using a concentration of 10 µM of sdAb in PBS (Dulbecco's Phosphate Buffered Saline). Increasing amount of hemin (stock dissolved in 0.1M NaOH and pH adjust to 7.4 with 0.1M HCl) was added and the incubated (5 min.) before recording the next spectra. Resonance Raman (RR) spectra were obtained using Raman spectrometer (Join Yvon U1000) coupled to a confocal microscope equipped with 1,200 lines/mm grating and a liquid nitrogen-cooled back-illuminated charge coupled device (CCD) detector. The spectra of 40 µM protein bound to heme and 50 µM heme in PBS buffer were obtained using a rotating cell (Hellma) to avoid photoreduction and measured with λ_{413nm} excitation line from a Krypton ion laser (Coherent Innova 302) with 4.5 mW laser power and 60 s accumulation time, at RT; typically 10 spectra were co-added to improve S/N ratio. After polynomial background subtraction, spectra were subjected to component analysis to determine the band-widths and positions, using in-house created software. Circular Dichroism (CD) Spectroscopy measurements were performed using a Jasco J-815 spectropolarimeter equipped with a Peltier-controlled thermostated cell support. CD spectra were recorded with sdAb 2H10 (240 µg/mL in 35% 50mM TRIS, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol; pH 7.4 in PBS). Spectra were collected (50nm/min.) and acquired (10x) to improve the signal to noise ratio. Attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectra were measured on a Bruker IFS 66/S spectrometer equipped with a MCT detector and a thermostatized Harrick BioATR II cell. All measurements were obtained using an ATR cell with sdAb 2H10 (700 µg/mL in 50mM TRIS, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol at pH 7.4) dissolved in PBS (1x), with and without heme. Each spectrum comprises the mean of 150 scans taken at a resolution of 4 cm^{-1}. Spectral assignments for specific secondary structure elements were made as in (35).

Immunofluorescence – HeLa (ATCC) cells, grown in DMEM supplemented with 10% fetal bovine serum (Life Technologies) and 1% antibiotics (Life Technologies),

were seeded onto coverslips (24 well plate) to reach 80% confluence 24h thereafter. Cells were washed (1x, PBS), incubated (5min., RT) in a hypotonic solution (85.5 mM NaCl, 5 mM $MgCl_2$, pH 7.0), fixed (ice cold methanol, 10 min., -20ºC or 4% paraformaldehyde for 10 min at RT), washed (1x, PBS), permeabilized (0.1% triton X-100 in PBS, 5 min.), washed (3x, PBS) and blocked (Protein-Free Blocking Buffer, Pierce, 1h, RT). For heme detection, HeLa cells were incubated with sdAb (2,5 ng/µL; 3h, RT), washed (3x, blocking buffer) and incubated (1h, RT) with Alexa Fluor ®647 conjugated HA-Tag (6E3) mouse mAb (1/200, Cell Signaling Technology). Cells were rewashed (3x, PBS, 1x milli-Q Water), dried and mounted in Mowiol-Dabco media with DAPI (Sigma). SdAb 2H10 was immunostained in combination with anti-Calnexin (integral protein of endoplasmic reticulum) Ab produced in rabbit (C4731, Sigma), anti-EEA-1 (localizes exclusively to early endosomes), phalloidin–tetramethylrhodamin.e B isothiocyanate phalloidin (a toxin that binds F-actin)(P1951, Sigma), anti-bilirubin mAb (A420, Dojindo) and anti-HO-1 polyclonal Ab (SPA-896, Stressgen). Mitochondria were stained with MitoTracker® Red (Life Technologies). Nuclei (DNA) were counterstained with DAPI. Images were acquired on a Leica DMRA2 upright microscope, equipped with a CoolSNAP HQ CCD camera, using a 100x 1.4NA Oil immersion objective, DAPI + FITC + CY5 fluorescence filter sets, controlled with the MetaMorph V7.5.1/ software. The analysis was done with ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

Flow Cytometry – HeLa cells were tripsinized (0.05% Trypsin-EDTA, Life Technologies), washed (1x, PBS), fixed (ice cold methanol, 10 min., -20ºC), re-suspended, washed (1x, PBS), permeabilized (0.1% triton X-100 in PBS, 5 min.), washed (1x, PBS) and blocked (Protein-Free Blocking Buffer; Pierce)(1h, RT). Intracellular heme was detected using anti-heme sdAb (2.5 µg/mL in blocking buffer, 2h30min., RT, shaking) followed by Alexa Fluor® 647 conjugated HA-Tag (6E3) mouse mAb (1/200, Cell Signaling Technology). Cells were washed (1x, PBS), re-suspended (PBS) and acquired in a FACScan (Becton Dickinson) with

CellQuest software (BD Biosciences). The analysis was done with FlowJo (Tree Star, Inc.).

Ascorbate oxidation assay – Ascorbate oxidation was used as a readout for oxidation activity of heme in the absence or presence of albumin or sdAb (48). Briefly, the reaction was initiated by the addition of heme (0-5 µM), without or with protein/sdAb (0-5µM) and hydrogen peroxide (800µM), to ascorbate (10 µM) in PBS pH 7.4 containing a metal chelator DETAPAC (50 µM) that does not inhibit heme mediated oxidation. The reaction was carried out under air at 37ºC and after a certain time, stopped by the addition of one volume of 0.5% metaphosphoric acid to one volume of reaction mix. The final solution was centrifuged and the supernatant collected and analyzed using HPLC with electrochemical detection (49)

Heme bound proteins preparation – Rabbit HPX (kindly provided by Ann Smith, University of Missouri, Kansas City, USA) (15) in PBS was incubated with hemin in DMSO at 1:2 molecular ratio (4h30 min., 4ºC, agitation). Non bound heme was removed by gel filtration chromatography (microcon YM-3; Milipore)(2x 30 min., 500uL PBS 1x) according to the manufacture instructions. SdAb in PBS was incubated with 2x molar excess hemin in NaOH/HCl pH 7.4 (ON, 4ºC). Non bound heme and free sdAb were removed using a PD10 column (GEHealthcare) pre-equilibrated in PBS. Heme binding to HPX or sdAb was confirmed spectrophotometrically. Concentration of HPX-heme complex was determined spectrophotometrically (λ_{414nm}; E_{mM}=141,6) (50). Concentration of sdAb-heme complex was determined using formic acid assay, as described above. Total amount of protein was determined using Quick Start Bradford Protein Assay (Biorad), according to manufacture instructions.

Heme depleted serum medium (HD) – The medium was prepared as described (51). Briefly, DMEM (Life Technologies) was supplemented with 10% heat inactivated fetal bovine serum (FBS; Life Technologies). Serum as pre-depleted from heme using ascorbic acid (10 mM; 37ºC, 50 rpm) until λ_{405nm}=0.6-0.8 (~7 h),

dialyzed (3x) in sterile PBS and filtered (0.2µm). Final culture medium was supplemented with 1% penicillin (10000 U/mL) and streptomycin (10000 µg/mL).

Golgi-HRP reporter assay was adapted from (37) – Briefly, HEK293 cells (ATCC; 5x104 cells/well in a 24 well plate) were grown overnight in DMEM, 10% FBS, 1% penicillin (10000 U/mL) and streptomycin (10000 µg/mL) and transiently transfected (4h-6h, lipofectamin 2000; Invitrogen) with an expression vector encoding the HRP gene under the control of the EF-1α promoter (pEF5/FRT/V5-DEST-Golgi-HRP) in opti-MEM reduced serum (Gibco) (37). HRP was confined to the Golgi with a targeting sequence from galactosyltransferase. Control HEK293 cells were transiently transfected with the pEF5/FRT/V5-DEST vector. Transfected cells were cultured in heme-deplete culture DMEM with or without 0.5 mM of succinylacetone (SA)(24h) and when indicated exposed to hemin, hemin-sdAb 2H10 or hemin-HPX (0.5 µM heme equivalents) in opti-MEM 0.5 mM SA (overnight). Peroxidase activity was assessed as a red-out of cellular heme content, given that the activity of the transfected HRP is dependent on the insertion of intracellular heme into apo-HRP. Briefly, cells were lysed (20 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2,5X protease inhibitor cocktail set; Calbiochem®)(ice, 20 min.), centrifuged (13.500 rpm, 4ºC, 5min.) and peroxidase activity was quantified in a 96 well plate using of 3,3',5,'-Tetramethylbenzidine (TMB) substrate reagent set (BD OptEIA). Reaction was stopped (2N H_2SO_4) and absorbance was measured in a microplate reader at λ_{450nm} (Vitor 3 luminometer). Peroxidase activity was determined based on the calibration curve generated with the serial dilution of HRP (type IV, Sigma) and normalized to the total amount of protein as determined using Quick Start Bradford Protein Assay (Biorad). HRP concentration was determined based on Beer-Lambert law using the extinction coefficient (λ_{403nm}; E_{mM}=100). Cellular heme uptake was determined based on the heme dependent peroxidase activity assay as describe previously, to generate a calibration curve using serial dilution of heme normalized to the total amount of protein as determined using Quick Start Bradford Protein Assay (Biorad).

Western blotting for HRP activity and protein detection – Protein collected from transfected HEK293 cells (50 µg) in loading buffer (20 % v/v Glycerol, 0.2 M Tris-HCl, pH 6.8; 0.05% w/v Bromophenolblue) was loaded onto 7% SDS-PAGE (50 µg) and transferred into polyvinyldifluoridine membrane, washed (1x, T-TBS) and HRP activity revealed immediately using peroxidase substrate (SuperSignal West Pico Chemiluminescent substrate) in a photoradiograph (Kodak Biomax Light Film; Eastman Kodak). Membrane was blocked (Protein-Free Blocking Buffer; 2-16h; RT) and re-probed with rabbit anti-HRP polyclonal Ab (Sigma, 1/2000; 1h, RT), washed (5x, T-TBS) and probed with Alakaline Phosphatase-conjugated goat anti-rabbit polyclonal Ab (Pierce; 1/5000; 1h, RT). Alakaline Phosphatase signal was detected using Lumi-Phos WB chemiluminescent Substract (Pierce) in a photoradiograph. Membranes were re-probed with anti-vinculin mouse mAb (1/3000, ON, 4ºC; Abcam) detected by HRP conjugated goat anti-mouse IgG polyclonal Ab (1/2500; 1h, RT). HRP signal was detected as described above. All Abs were diluted in Protein-Free Blocking Buffer.

Primary hepatocytes isolation – The isolation of Primary hepatocytes was performed as described (15). Briefly, the right liver lobe was extracted from 10-12 weeks C57BL/6 mice and perfused through the portal vein with liver perfusion medium (20 mL; 4mL/min.; Gibco) and liver digestion medium (20 mL/lobe; 4mL/min.; Gibco). Liver was disrupted in liver perfusion medium, filtered (100 µM strainer) and collected (130g, 60 sec, RT, no break) in liver perfusion medium. Cells were re-suspended (24 mL) in William´s E medium (Gibco) supplemented with 4% FBS, 1% penicillin (10000 U/mL) and streptomycin (10000 µg/mL) and added (6 mL) to a 1.06 (5 mL)/1.08 (5 mL)/1.12 (4 mL) g/mL Percoll gradient (750g, 25 min., RT, no break)(Amersham, Buckinghamshire, UK). Hepatocytes were collected, washed twice (1x 40 mL, 1x 10 mL; 200g, 10 min., 4ºC) and re-suspended in William's complete medium. Hepatocytes were seed in a gelatin (0.2%) coated plated and experiments performed 24 h thereafter.

Cytotoxic assay – The cytotoxic assay was performed essential as described (15). Briefly, primary hepatocytes were seeded ($3.5x10^4$ cells/well; 24 h) in a 96 well plate and exposed to heme, sdAb-heme or HPX-heme (5 µM heme-equivalent, 1 h, 37ºC) in Hank´s Balanced Salt Solution (HBSS, Gibco). When indicated hepatocytes were exposed mouse recombinant tumor necrosis factor alpha (TNF, R&D Systems)(5 µg/mL; 16h, in William's complete medium. Cell viability was assessed by crystal violet assay (15).

Mice – Experiments in mice were performed in accordance with protocols approved by the Ethics Committee of the Instituto Gulbenkian de Ciência and the Portuguese National Entity (DGAV Direção Geral de Alimentação e Veterinária) and with the Portuguese (Decreto-Lei nº 113/2013) and European (Directive 2010/63/EU) legislation related to housing, husbandry and animal welfare. C57BL/6 mice were bred and maintained under specific pathogen-free conditions under ethical approval by the Instituto Gulbenkian de Ciência animal care committee.

Animal samples – Mouse plasma was obtained by cardiac puncture under heparin (EDTA anti-coagulated blood), centrifuged (2x; 1600g, 5min., 4ºC). Samples were maintained at -80ºC until be used.

Phenylhydrazine (PHZ) treatment – Age matched (10-12 weeks) male C57BL/6 mice were injected (s.c.) with freshly prepared phenylhydrazine hydrochloride (Sigma)(1xPBS, pH7.4; 90 mg/Kg; BW).

Total heme measurement – Performed essential as described (52). Briefly, samples were diluted in H_2O in 96 well plate and heme concentration was determined by comparison to a hemin standard curve (0.5-16 µM in H_2O). Formic acid (150 µL/well; 98-100%, Merck) was added and absorbance measured at λ_{405nm} using a microplate reader (Vitor 3 luminometer).

Statistical Analysis – In the comparison of the means of more than two groups, we used the analysis of variance (ANOVA) and significance between groups was estimated using Bonferroni post-test, when Gaussian distribution was observed. For samples with non-Gaussian distribution, comparison between groups was performed using a Kruskal-Wallis test, followed by post test Dunns to compare all pair groups. Statistical analysis were performed using the GraphPad v.5.0a software (Prism), and p values were represented as *$p < 0.05$, ** $p < 0.01$, ***$p<0.001$

6. ACKNOWLEDGEMENTS

Dr. Ana Maria Varela Coelho and Renata Soares at Mass spectrometry and analytic services units of the Instituto de Tecnologia Química e Biológica (ITQB). Pierre Crozet and Jorge Carneiro for help in analysis of HBC. Dr. C Suarna for help with the ascorbate oxidation studies. The entire inflammation group at Instituto Gulbenkian de Ciência for critical discussion and comments. This work was supported by Fundação para a Ciência e Tecnologia (RECI-IMI-IMU-0038-2012; PTDC/SAU-TOX/116627/2010; HMSP-ICT/0018/2011 to MPS, SFRH/BD/44828/2008 to ZG, SFRH/BPD/47477/2008 to SSL, PTDC/SAU-FAR/119173/2010 to JG), from ERC-2011-AdG 294709-DAMAGECONTROL to MPS, and NHMRC Senior Principal Research Fellowship 1003484 to RS.

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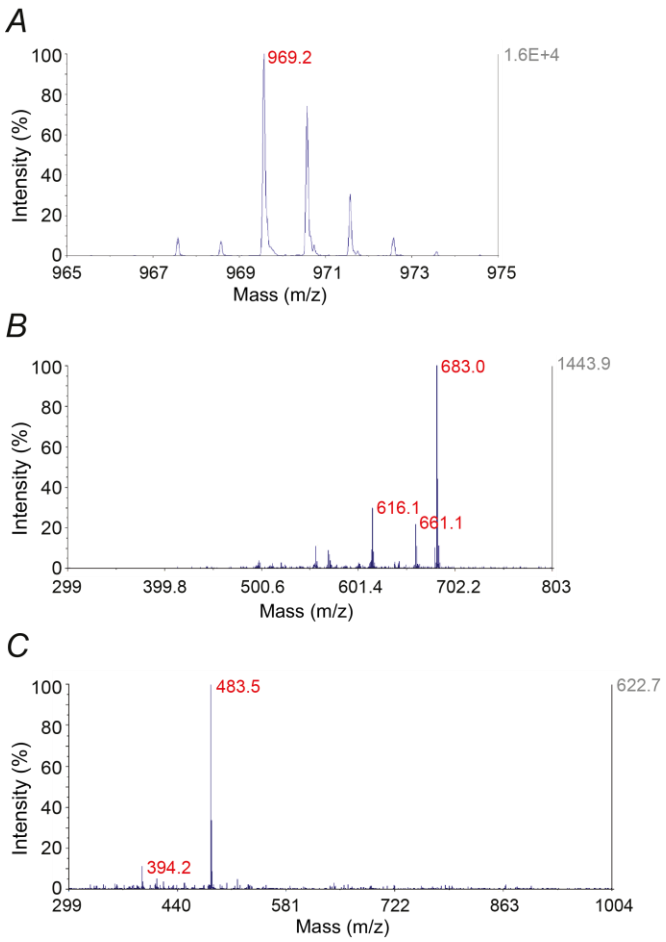
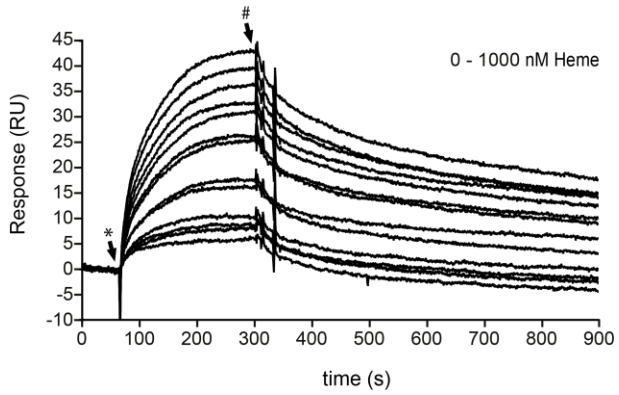
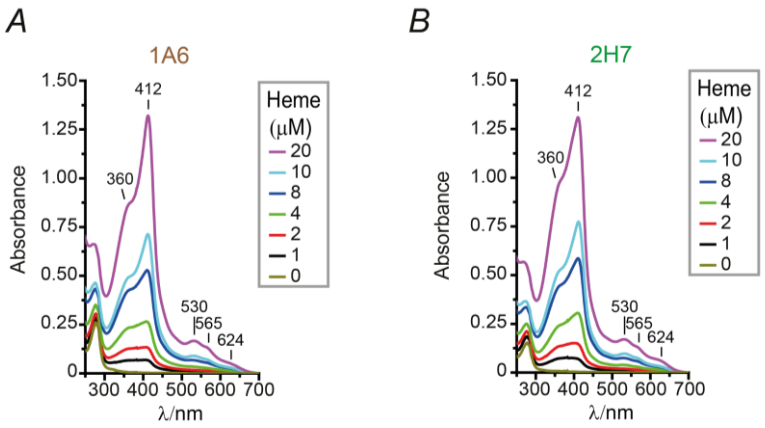
8. SUPPLEMENTARY FIGURES



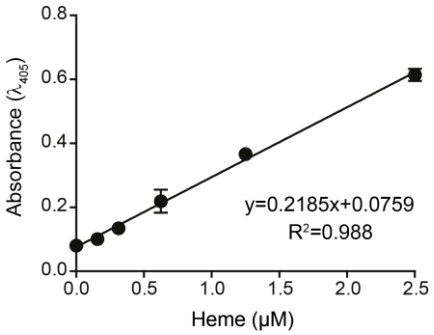
Suppl. Figure 1. MALDI-TOF/TOF zoom analysis of biotinylated heme. (A) Mass-to-charge (m/z) 969.2 Da showing an isotopic cluster pattern similar to the theoretical pattern calculated for the heme-biotin molecule in which a single carboxylic acid residue of heme is biotinylated. (B) MALDI-TOF/TOF analysis of hemin. Mass-to-charge 683.0 Da. (C) MALDI-TOF/TOF analysis of biotin. Mass-to-charge of 483.5.0 Da.



Suppl. Figure 2. Sensogram of heme binding to sdAb 2H10. For the determination association and dissociation kinetic rate constants, used to calculate the constant of affinity (KD) from the sdAb was immobilized in a CM5 chip using using amine coupling at ~800 resonance units (RU) and different concentration of heme (from 0 to 1000 nM) infected for 4 min. The symbol * indicates the start and # the end of heme infection.



Suppl. Figure 3. Characterization of heme binding to sdAb 1A6 and 2H7 by UV-Visible spectrophotometry. UV-Visible spectra of sdAbs (A) 1A6 and (B) 2H7, pre-incubated with increasing concentrations of hemin. Soret (412 nm), Q_1 (530 nm), Q_0 (565 nm) and CT (624 nm) bands are highlighted.



Suppl. Figure 3. Sandwich ELISA for heme detection. Soluble hemin was captured by sdAb 1A6 and revealed by sdAb 2H7. Data shown is representative of three independent experiments.

CHAPTER 3

GENERAL DISCUSSION

Regulation of Fe metabolism is critical for maintenance of homeostasis (Gozzelino and Soares, 2014). In mammals, 75-80% of bioavailable Fe is contained inside heme and the remaining 20-25% exists in the form of non-heme (labile) Fe (Gozzelino and Soares, 2014). Around 80% of total heme is contained in the erythroid lineage where it is used to produce hemoglobin, while 15% is present in liver and the remaining in other tissues. Several pathologic conditions are associated with variation of homeostatic parameters that are incompatible with the maintenance of RBC integrity thus leading to hemolysis and to the release of hemoglobin into plasma. The shear number of total RBC ($2\text{-}3x10^{13}$ in humans) combined with their high hemoglobin content ($3x10^{8}$ molecules/RBC) and the corresponding heme amount ($1.2x10^{9}$ molecules/RBC) makes that clinically undetectable levels of hemolysis are associated with the release of significant amounts of hemoglobin into plasma. Tetramers of cell-free hemoglobin dissociate into dimers when oxidized (Bunn and Jandl, 1968;Hebbel et al., 1988;Balla et al., 1993;Pamplona et al., 2007) releasing their non-covalently bound heme *b*. The non-hemoglobin bound heme is commonly referred as “free heme” (15-16). However, heme is most probably never found as a free molecule, being bound to a series of heme binding molecules, including proteins and lipids. Heme binding proteins in the plasma include hemopexin (Tolosano et al., 2010), albumin (Fanali et al., 2012) and lipoproteins (Camejo et al., 1998). Along this work, we use the term “free heme” to refer to labile, redox active heme which is loosely bound to proteins or other molecules but can presumably act in a deleterious manner.

Several studies suggest that once released from hemoglobin, heme *b* can act in a vasoactive (Adisa et al., 2013;Vinchi and Tolosano, 2013;Belcher et al., 2014), pro-inflammatory (Graca-Souza et al., 2002;Figueiredo et al., 2007) and cytotoxic manner (Seixas et al., 2009;Gozzelino et al., 2010;Larsen et al., 2010) promoting the development and progression of immune mediated inflammatory diseases, such as sickle cell anemia (Nagababu et al., 2008;Vinchi et al., 2013), malaria caused by *Plasmodium* infection (Pamplona et al., 2007) or severe sepsis caused by polymicrobial infections (Larsen et al., 2010;Gozzelino et al., 2012). Therefore, mechanisms controlling heme homeostasis are likely involved in the pathogenesis

of these diseases and probably others (Schaer et al., 2013;Gozzelino and Soares, 2014). While the pathologic effects of “free heme” are most probably associated with its release from cell-free hemoglobin, we cannot exclude that other sources of heme might act in a similar manner, such as the heme released from myoglobin during the process of rhabdomyolysis (Nath et al., 1992). Current limitations in the methodological approaches allowing qualitative and quantitative analyzes of heme, when released from hemoproteins, have hindered the capability to determine unequivocally its pathologic effects as well as an eventual therapeutic targeting. This PhD Thesis aimed to overcome such limitations through the development of methodologies that would allow the qualitative and quantitative analyses of heme in biologic system.

Abs have been extensively used for the development of bioassays (Engvall and Perlmann, 1971;Konishi et al., 1985) as well as for targeting potentially pathogenic molecules (Leavy, 2010), aiming to enhance human health (Elvin et al., 2013). Phage display technology has been recognized as a very powerful approach in the development and selection of Abs directed against a wide range of antigens (**Chapter 1 Fig. 14 and 17**) (Winter et al., 1994;de Kruif et al., 1995;Boel et al., 2000;Benhar and Reiter, 2002;Gargir et al., 2002;Binyamin et al., 2003;Silacci et al., 2005;da Silva et al., 2008;Kim et al., 2008;Beer and Liu, 2012). Another important advantage related to this technique is the possibility to make an *in vitro* screen instead of *in vivo,* which is conventionally used in other antibody generation techniques. This does not require special equipment and has a higher flexibility and versatility in terms of the selection conditions and stringency (Hoogenboom, 1997;Watters et al., 1997;Rodi et al., 2002). Once performed *in vitro*, the selection can be performed against biological as well as inorganic compounds (Whaley et al., 2000;Kriplani and Kay, 2005). This was a crucial aspect to raise Abs against “free heme”. In addition, the traditional techniques for antibody generation rely on the injection of the antigen into a host, *i.e.* mouse, rat or rabbit and thus are to some extent limited by the toxicity of the antigen. As the administration of high dose of “free heme” are known to be cytotoxic (Larsen et al., 2010;Gozzelino and Soares,

2011), compromising the viability of the host, we use of phage display technology to generate Abs against heme.

We decided to raise single domain Abs (of small size; about 15 kDa) against heme which can be generated based on the VH or VL domains of a classical antibody (**Chapter 1 Fig. 16**) and can be genetically manipulated thereafter. The single domain format offers several advantages when compared with conventional antibodies and other recombinant antibody fragments as the single-chain antibody (scFv) (Holt et al., 2003). These include higher capacity to recognize rather small epitopes in antigens or the ability to bind the active site of enzymes. *In vivo*, they were shown to have higher capacity of tissue penetration (da Silva et al., 2008), allowing their access to antigens localized or hidden in region difficult to reach by conventional antibodies. Production of sdAb can reach high levels without loss of solubility, be produced in different systems including bacteria. This makes their production simpler, faster and cheaper, as compared to full length antibody with limited expression efficiency in *E. coli* expression system (Mazor et al., 2007). Furthermore, the therapeutic applications of sdAbs were already demonstrated in several diseases (Harmsen and De Haard, 2007;da Silva et al., 2008;Wesolowski et al., 2009) and therefore we rationalized that this technology might offer a good alternative to more classical methods used for antibody production to target “free heme” *in vivo* (**Chapter 2 Fig. 1**).

The development of sdAbs against heme could be further used to develop methodologies that would allow “free heme” quantification in biological system as well as its cellular detection. Recently, the VL single domain type has been described as a suitable alternative, although, the most common type of sdAbs used is VH single domain due to its well documented properties, such as high stability, production levels and binding efficiency. The first VL sdAbs with fully binding activity against huntingtin, a protein associated with the pathology of Huntington's disease were described and developed in 2004 by Colby and co-workers (Colby et al., 2004). These sdAbs were presumably more stable and active than the associated VH single domain, suggesting that this type of single VL domain antibodies can be more efficient against certain antigen. This study provided evidences supporting the

usage of this type of domain, at least against certain type of antigens. This was further supported by other studies (Brinkmann et al., 1993;Kim et al., 2014). Therefore, as a first trial, we used a synthetic VL sdAbs phage display library for the selection of sdAbs against heme. Nevertheless, the VH single domain was also considered in case that no VL- sdAbs could be raised against heme. Amongst the different types of phage display libraries (da Silva et al., 2008) we decided to use a synthetic library of sdAbs since it allows the exact construction and controlled genetic manipulation of paratope sequences that are not present in the germline antibody repertoire. This is expected to provide higher and/or more stringent diversity amongst the sdAbs, therefore allowing to guide the sdAbs selection against new or challenging epitopes, such as heme, which are conceivably hard to select from naïve Abs. Besides that, these sdAbs frameworks can be genetically manipulated to decrease or inhibit sdAbs immunogenicity (*e.g.* by humanizing frameworks) and to improve not only the sdAbs stability but also the expression yields in different systems, such as bacteria.

The VL synthetic library used in the sdAbs selection against heme has a high diversity range of 10^9 variants and was previously tested successfully to raise sdAbs against other targets, such as viral gp41 in HIV (Gonçalves *et al*, submitted) (**Chapter 2, Fig. 1B and 1C**). From these phage display screens, we were able to select and purify around eleven heme-binding sdAbs (**Chapter 2, Fig. 1C and 1B**). These sdAbs typically showed high expression and solubility in bacterial systems, and we could verify that the selected sdAbs had a high expression yield. However they displayed low solubility as assessed by their association with inclusion bodies. In order to improve the sdAbs solubility, we modified several expression conditions known to improve protein solubility in bacteria. These include different bacteria growth temperature, media, expression plasmid and strain; however no improvement was observed (*data not shown*). Consequently, we resorted to a purification method under denaturing conditions to collect sdAbs from the bacterial inclusion bodies and then processed to their renaturation. The denaturation approach used was based on a standard method in which the proteins are isolated from inclusion bodies by a series of sonication and centrifugations steps, followed

by solubilization using higher concentration of a solubilizing agent (6 M urea). These sdAbs contain cysteines in their backbone, therefore for the complete protein denaturation, it is highly recommended to add a reducing agent, such as β-mercaptoethanol, for cysteine reduction and disulfide bond cleavage. The sdAbs were purified using a His trap column, which allows obtaining a high yield and a reasonably good quality of purified protein. We could also use a HA tag column to have a more pure protein, however the yield obtained is lower and the anti-HA antibody is expensive. Although we successfully obtained a higher yield of purified protein, the next step: protein folding, revealed to be the major challenge. We were confronted with substantial protein precipitation (> 90%) upon refolding, employing typical and widely used method for fast removal of the denaturing buffer to non denaturing, which promote protein folding (such as PD10 desalting column or Protein desalting spin columns). Therefore, we decided to use a step-wise dialysis method that although time consuming (several days) allows for protein folding. This step-wise dialysis protocol was slightly modified based on previously described methods (Tsumoto et al., 1998;Umetsu et al., 2003;Tsumoto et al., 2004;Tsybovsky et al., 2007). These included the initial removal of the reducing agent to promote thiol groups oxidation and consequently disulfide bond formation, followed by sequential overnight dialysis with decreased concentration of the solubilizing agent (urea) until complete removal and medium supplemented with additives, namely L-arginine and oxidized/reduced glutathione in the antepenultimate and penultimate dialysis steps. The use of additives in the dialysis revealed to be crucial to promote protein stabilization and refolding (Tsumoto et al., 1998;Umetsu et al., 2003;Tsumoto et al., 2004;Tsybovsky et al., 2007). Presumably, the beneficial effect of L-arginine relays in its capacity to interact with certain amino acids in the proteins, namely to aromatic side chains and predominantly to Tyr and Trp, leading to an increase of protein surface tension of water (Arakawa et al., 2007;Das et al., 2007). This effect presumably prevents protein/protein interaction and aggregation (Arakawa et al., 2007;Das et al., 2007). On the other hand, the redox GSSG/GSH pair apparently promotes the folding of protein by thiol (RSH)-disulfide (R'SSR') interchange reaction, leading to the formation of disulfide bonds and most probably

to the rearrangement of the secondary and tertiary protein structure. Further studies are required to better understand the molecular mechanisms that lead to their effectiveness. In this work, the improvement of the process of protein purification and recovery allowed us to obtain high yields of protein folding (>90%) with higher purity (**Chapter 2 Fig. 1B**), which was crucial to perform this project.

The purified sdAbs were analyzed for their ability to bind heme, using an ELISA protocol in which hemin was coated on the plate. All the sdAbs bound heme in to a similar extent, with exception of sdAb 1E4. The negative control, which was a sdAb with the same backbone but raised against an irrelevant antigen, showed no binding, supporting the specificity of the sdAbs raised against heme. We analyzed further the binding of these sdAbs to heme using BIAcore which is a surface plasmon resonance (SPR) based technology, highly used in the studies of biomolecular interactions such as protein-protein, protein-DNA and allowing for the determination of their affinity parameters (Stenlund et al., 2006;Rich and Myszka, 2008). The determination of heme binding affinity of the sdAbs allowed us to make a comparative study to other heme scavengers with known heme binding affinity constant (K_D), such as hemopexin ($K_D=10^{-12}$M) (Shipulina et al., 2000) and others with lower affinity such as human serum albumin ($K_D=10^{-8}$M) (Adams and Berman, 1980) and α_1-microglubolin ($K_D=10^{-6}$M) (Larsson et al., 2004). The heme binding curves, shown for sdAb 2H10 (**Chapter 2 Suppl. Fig. 2**) are representative of those obtained for all sdAbs and reveal that a fast on rate (association) for heme is followed by its considerable rapid rate of dissociation, suggesting that rapid “in and out” kinetics for heme. From a putative therapeutically perspective, the fast release of heme might be advantageous by facilitating heme excretion through the urinary track and/or for its metabolism by HO-1, contrarily to heme binders with a slow or limited dissociation rate. However, if desirable, the rapid dissociation constant can be limited or controlled though a more frequent and/or more concentrated sdAb administration. We could determine the heme affinity constant (K_D) of the sdAbs as the association and dissociation constants fitted in a kinetic model of 1 to 1, which revealed that all the sdAbs bind heme with a similar affinity of 10^{-7} M with exception of sdAb 1E4 with the value of 10^{-5} M (**Chapter 2 Fig. 1F**). When compared with the

known extracellular heme binding protein, we verified that the sdAbs have a lower affinity than the major and most abundant known extracellular heme binding proteins in plasma: hemopexin, lipoproteins, human serum albumin, but higher than α_1-microglobulin, suggesting that the sdAbs can only bind heme when it is not bound to those proteins. Therefore, sdAb most probably bind to heme only when it is bound to other proteins that have a heme binding affinity lower than 10^{-7}M or when present in free form, which is unlikely to occur *in vivo* (**Chapter 2 Fig. 1**). We also need to consider that the sdAbs might not recognize heme in the hemoproteins because it is contained inside of them and therefore not accessible to the sdAb.

Hemoproteins and heme scavengers contain a sequence of key amino acids that interact with heme, referred as Heme Binding Motifs (HBM). Amongst the most common HBM demonstrated to bind heme *b*, there are two recently proposed HBM Cys-Ser (CS) and Cys-Asn (CN) (Yang et al., 2010;Westberg et al., 2011) that we found in the region of heme recognition, in the CDR1 amino acid sequence of the sdAbs. The CS is present in sdAb 2H10 and CN in 2H7 (**Chapter 2 Fig. 1F**), which most probably participate in heme binding. In the sdAbs 2H10, there are other amino acids beside cysteine that might be involved in its binding to heme, namely tyrosine and lysine, as it is known that in certain hemoproteins tyrosine and lysine can participate as heme *b* axial ligands (Ferrer et al., 1993;Schneider et al., 2007;Li et al., 2011). We also identified a HBM correlated to the covalently binding of heme *c* in cytochrome P460, namely the CXXCK, in the sdAb1A6 (**Chapter 2 Fig. 1F**). However, this motif is most probably not correlated to the binding of heme to the sdAbs, since this binding requires the enzymatic activity of a chaperone to catalyze the thiol attachment of cysteines to the vinyl groups of heme as demonstrated in bacteria, archaea and plants (Verissimo et al., 2013). Instead, we propose a new HBM composed of the amino acids CK, responsible for heme *b* binding in this sdAbs. This hypothesis is supported by observation that in several hemoproteins both amino acids cysteine and lysine can act as axial ligands of heme-Fe (Ferrer et al., 1993;Rosell et al., 1998;Li et al., 2011). We however did not explore this hypothesis and thus further studies are required. This might include the construction of a range of mutated sdAbs by site directed mutagenesis at the levels

of the amino acids previously proposed to be in the HBM, followed by their functional analysis by ELISA, heme-biotin pull-down or BIAcore. Additionally, spectroscopic studies can be done in a similar manner to what was performed for the non mutated sdAbs (Wilks, 2002). Otherwise, we can collect detailed structural information on the original sdAbs bound to heme *via* Nuclear Magnetic Resonance (NMR) spectroscopy and X-ray crystallography, and thus further elucidate the structure-function of sdAb-Heme (Wilks, 2002). Furthermore, these techniques will allow us to identify the amino acids interacting with heme *b* and details of this interaction occurs. This might provide insights, once compared to genomic data, to the range of proteins that contain HBM.

Another important characteristic of the sdAbs developed under this PhD work is the absence of the CDR3 region, even though the library used to select sdAbs against heme contains diversity in the range of 10^9 in which the variability is restricted to the antigen recognition CDR1 and CDR3 amino acid sequence in VL domains. During phage display screen against heme, there was an enrichment of sdAb that do not contain diversity in CDR3 amino acids sequence. We cannot fully explain why the selected sdAbs do not have this CDR3 sequence. However, preliminary results (*data not shown*) suggest that the initial library contained diversity of sdAbs with variability in CDR1 and CDR3 or only in their CDR1, therefore during the pannings the sdAbs containing variability in CDR1 were enriched while the other were removed. The diversity in this synthetic library, *i.e.* at the levels of CDR sequence and composition was most probably generated during the library construction. Diversity was generated in two different steps, first randomization in CDR1 and then in the CDR3 region; therefore it is possible that in some of the sdAb the diversity in the CDR3 was not successfully achieved (Gonçalves et al, submitted) and consequently some of the sdAbs only have diversity in their CDR1. This however was not investigated, hence further experiments are required and possibly re-calculation of the library diversity considering not only the diversity in the CDR but also the presence/absence of CDR3. The enrichment of sdAbs against heme without the CDR3, might be justified

by a higher sdAb stability and affinity upon heme binding or that the affinity of the sdAbs is negatively affected by the presence of the CDR3.

We selected three sdAbs (1A6, 2H7 and 2H10) that recognize heme, of which sdAb 2H10 appears to be the most specific (**Chapter 2 Fig. 1, 2 and 3**). These sdAbs have different binding properties for heme (**Chapter 2 Fig. 2 and 3; Table 1**) that might be correlated with their different HBM in the CDR sequence., namely CXXCK or most probably CK for sdAb 1A6, CN for sdAbs 2H7 and CS (or CK; CY) for sdAb 2H10 (**Chapter 2 Fig. 1F**). Although, all the three sdAbs have a similar heme binding affinity, their interaction with heme is established by different heme epitopes. Our results shown that sdAb 1A6 interacts with heme independently of the presence of Fe and the availability of the propionate groups (**Chapter 2 Fig. 2 and 3; Table 1**). This suggests an interaction with heme independent of the vinyl groups as well as the propionate groups, so other regions in the heme should interact with the sdAb. Further studies are required to understand this interaction and whether the presence of the CXXCK motif in the sdAb is required for heme interaction. The sdAbs inability to bind ZnPP might be correlated with its physicochemical properties. Indeed, this metal is commonly found coordinated with four ligands, establishing a tetrahedral molecular geometry and can only acquire an oxidation state Zn^{2+} and thus usually lacking redox activity. This might confer some inertiness to the protoporphyrin thus limiting its protein binding (Pace and Weerapana, 2014). On the other hand, the sdAb 2H7 can interact with ZnPP but not with empty PP, suggesting that the binding requires its interaction with central metal in the PP (**Chapter 2 Fig. 2 and 3; Table 1**). It was demonstrated that cysteines can coordinate with Fe but also with Zn and Co (Gavel et al., 2008;Pace and Weerapana, 2014). It is therefore possible that the cysteines are part of the HBM and can be coordinated with the Fe as well as with other metals in the protoporphyrin. The sdAbs 2H10 differs from the other sdAbs by its inability to bind to empty PP, ZnPP and to heme analog with propionate groups blocked by a methyl group, indicating that the binding to heme requires the simultaneous interactions of the sdAb with the Fe and the propionate groups of heme (**Chapter 2 Fig. 2 and 3; Table 1**). We propose the cysteine of the HBM as one of the axial

ligands and lysine or tyrosine as another (**Chapter 2 Fig. 4**), however further studies are required to assess which amino acids interact with the propionate groups. We conclude from these assays that the sdAb 2H10 is the most specific for heme, a result that is further supported by its lack of interaction with the heme degradation products, biliverdin and bilirubin (**Chapter 2 Fig. 2; Table 1**). We also verified that this sdAb failed to recognize heme when contained within several hemoproteins (hemoglobin, myoglobin and cytochrome c) or even when bound to the heme scavenger, human serum albumin (**Chapter 2 Fig. 3**). This property of the sdAb prevents a possible toxic effect, if used *in vivo*, possibly caused upon binding to heme in hemoproteins that would presumably impair their biological function. Amongst several hemoproteins, we decided to test sdAb binding to hemoglobin and myoglobin as certain immune mediated inflammatory diseases are associated with hemolysis and accumulation of cell free hemoglobin in plasma (Pamplona et al., 2007;Larsen et al., 2010;Ferreira et al., 2011) or with rhabdomyolysis and myoglobin accumulation in plasma (Adornato et al., 1978;Nath et al., 1992). Besides that, both proteins can release their non-covalently bound heme *b*, presumably contributing to heme accumulation in plasma during those pathologic conditions and thus exacerbate disease severity (Adornato et al., 1978;Pamplona et al., 2007;Larsen et al., 2010;Ferreira et al., 2011). We also tested cytochrome c because it contains heme *c* covalently bound to the protein. This hemoprotein is normally present in the outer surface of the inner mitochondrial membrane, however, in certain pathologies cytochrome c can be released into the cytosol, inducing caspase activation and subsequent apoptosis, followed by its release into blood (Jiang and Wang, 2004). Whether this effect is heme dependent is not clear, but this is likely the case given that the cytochrome c redox activity is required to support its pro-apoptotic effect (Pan et al., 1999;Suto et al., 2005;Borutaite and Brown, 2007). Moreover, cytochrome c also accumulates in plasma in several pathologies, including systemic inflammatory syndrome (Renz et al., 2001;Adachi et al., 2004). The other protein tested was the heme scavenger albumin, the most abundant protein in the plasma, which binds heme transiently with one of the lowest heme binding affinity amongst the known heme scavengers

(**Chapter 1 section 4**) (Adams and Berman, 1980). Therefore, heme released from albumin should be more prompt to occur and then to be transferred/bound by sdAbs, for instance upon administration *in vivo*. However, this was not the case, suggesting that the sdAbs can only bind heme when it is in its "free" form or bound to heme binding proteins with an affinity for heme lower than 10^{-7}M, such as α_1-microglobulin. We did not explore the ability of the sdAbs to bind heme in α_1-microglobulin, because this protein is not commercially available and its expression/ purification is challenging.

In addition from testing the ability of the sdAs to bind heme immobilized into a solid surface (ELISA plates) (**Chapter 2 Fig. 1E, 2 and 3**) we confirmed the ability of these sdAbs to bind heme in solution, using a pull down assay with biotinylated heme (**Chapter 2 Fig. 3A**). To further characterize sdAbs binding to heme we applied spectroscopic methods, such as UV-Vis absorption spectroscopy, circular dichroism (CD), Fourier transform infrared (FTIR) and resonance Raman (RR). The use of these complementary techniques, allowed obtaining valuable information on the nature of the heme coordination, ligation and oxidation state as well as modification in the secondary structure of the sdAb 2H10. Briefly, UV-Vis absorption spectroscopy showed a characteristic UV-Vis features which originate from heme-protein interaction. These include an intense band at around 400 nm, the so-called Soret band, and less intense bands (δ, Q_0, and Q_1 bands), providing information on Fe oxidation state. While the CD in far-UV region (>250 nm) gives important information about the secondary structure of the protein, the near in UV-Visible region provides structural information related to the integrity of the cofactor binding sites, such as heme in the proteins. We also employed vibrational spectroscopies, , FTIR that provides more detailed information of the secondary structure of the protein and RR spectroscopy, which upon excitation using 413 nm laser light reveals information specifically about the heme chromophore, its structural properties, such as coordination and spin state. We could conclude that heme bound to sdAb 2H10 is in a six coordinate low spin state; most probably involving a cysteine as the fifth ligand and possibly lysine or tyrosine as the sixth axial ligand (**Chapter 2 Fig. 4B-E**) (Li et al., 2011). Upon heme binding the secondary structure

of sdAbs composed mainly by anti-parallel β-sheets, undergoes conformational modification, generating new α-helices and/or disordered segments (**Fig. 1**). However, further studies are required to elucidate the complete structure of this complex, using, as previously mentioned, site direct mutagenesis followed by the several techniques that will provide detailed structural information (*e.g.* NMR spectroscopy or X-Ray Crystallography).

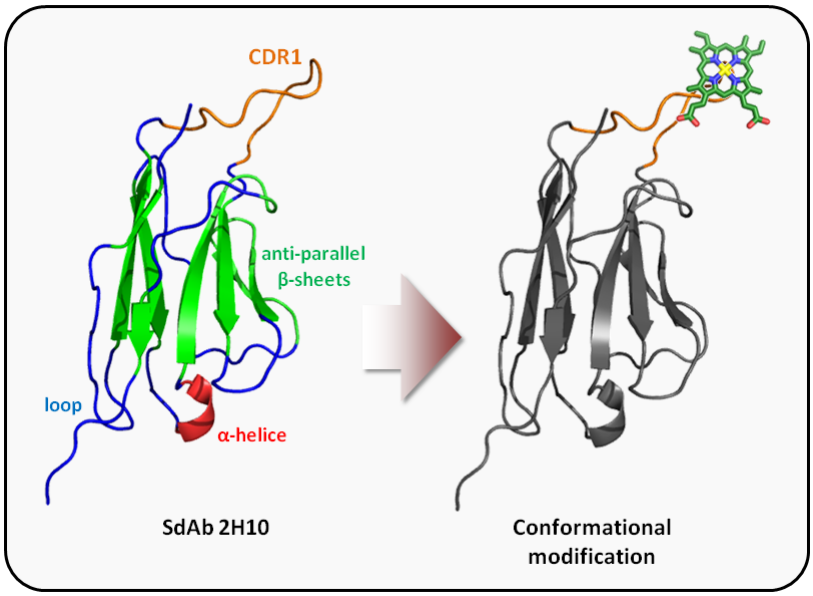


Figure 1: 3D model structure of sdAb 2H10. This 3D structure was generated based on the amino acid sequence of the sdAb 2H10 against heme using SWISS-MODEL software (http://swissmodel.expasy.org/interactive).In CDR1 are highlighted in orange.

Having shown that the sdAbs can bind heme, we then investigated their ability to detect heme *in vitro* by immunofluorescence microscopy (**Chapter 2 Fig. 5A, D, F and 6**) and Flow cytometry (**Chapter 2 Fig. 5B, C, E**). Both techniques could be successfully applied to detect endogenous heme in human HeLa cells (**Chapter 2 Fig. 5 and 6**). We could show using the most heme specific sdAb 2H10 that “free heme” is mainly localized in the mitochondria (**Chapter 2 Fig. 5F**), while a small portion is in the endoplasmic reticulum (ER), (**Chapter 2 Fig. 6B**). This suggests

that cellular heme is predominantly found in the mitochondria where it is synthesized, while only a small portion of “free heme” is found in the ER where we can speculate that some heme is incorporated into the heme pocket of folded proteins (Chapman et al., 1997;Poulos, 2007;White et al., 2013). We also verified that cellular heme was localized in close vicinity of HO-1 (**Chapter 2 Fig. 6D**) and to a less extent in the vicinity of bilirubin (**Chapter 2 Fig. 6E**), a secondary degradation product of heme catabolism produced by the biliverdin reductase (**Chapter 1 Fig. 9**). These observations support the cellular localization of “free heme” in the region where it is catabolized by HO-1, where the substrate, enzyme and end-product can be found. We cannot exclude that heme might be localized in other organelles which were not tested in this work, including Golgi apparatus, exosomes, ribosomes amongst other, therefore allowing to better elucidate the cellular localization of heme and its biological role.

As mentioned in the **chapter 2 section 4**, we cannot exclude the possibility that the cellular heme detected by the sdAb is heme that was released by hemoproteins denaturated during cell treatment, namely by methanol and/or detergent, used in immunoFluorescence/Flow Cytometry assays. Moreover, this treatment might also induce conformational changes in the protein structure, exposing their heme group thus recognizable by the sdAb.

Heme cellular localization in different cell, such as red blood cells, renal cells, immune cells and other cell types should be address under normal, hemolytic or other pathologic conditions in which heme might be involved. This information will help to elucidate the pathologic effect exerted by cellular heme accumulation (**Chapter 2 Fig. 7B**), for example in pathologic conditions associated with hemolysis. We cannot exclude however, that these sdAbs may react with heme that is loosely or transiently bound to cellular proteins or to other biologic molecules with an affinity higher than 10^{-7} M.

We further investigated whether we could use these sdAbs to develop methodologies allowing to overcome the current limitations in “free heme” detection and quantification, upon heme accumulation in plasma and/or tissues during immune mediated inflammatory disease associated with hemolysis As previously

mentioned in the **chapter 2 section 4**, the methods developed so far for “free heme” quantification have several limitations that compromise their efficiency and sensitivity in “free heme” detection. These include the method described in (Pamplona et al., 2007;Seixas et al., 2009), in which the plasma is filtered to remove proteins with a molecular weight higher than 3 kDa, followed by “free heme” quantification using a chromogenic assay. However, it cannot be excluded that in the collected fraction, there are no proteins or other molecules with low size (below 3kDa) and heme binding capacity. Moreover, if heme is present as “free”, it can be retained by the filter used. On the other hand, the method used in (Larsen et al., 2010;Gozzelino et al., 2012;Jeney et al., 2014) takes advantage of the peroxidase activity of heme alone or when associated to other molecules to measure total heme (Grinberg et al., 1999) as well as the UV-visible absorption of hemoproteins for hemoglobin quantification, allowing to access the amount of non-hemoglobin bound heme, *i.e. “*free heme” in plasma. The major limitations of this method are the variability of the peroxidase activity of heme upon its binding to the different molecules as well as the spectra of UV-visible of plasma is not specific for hemoglobin (Brill and Sandberg, 1968;Kuzelova et al., 1997;Zijlstra and Buursma, 1997;Mauk et al., 2007;Nicoletti et al., 2008). For instance, the spectra of all hemoproteins or even heme scavengers have common features (soret, Q bands and CT bans) and for instance the peaks of spectra can vary with buffer, heme ligands and oxidation state.

Therefore, the development of methodologies allowing “free heme” detection is crucial to understand the role of heme in the pathogenesis of those diseases. For that purpose, we adapted a well known and widely used biochemical assay, the sandwich ELISA, for heme quantification using the sdAbs (**Chapter 2 Suppl. Fig. 3**). The assay was designed to have one of the sdAb as a capture antibody and another biotinylated sdAb with different heme binding properties, as an antigen detecting antibody. We used the sdAb 2H7 as the biotinylated antibody. We used this clone because biotinylation of an amide bond occurs at the primary amino groups of the amino acids, such as lysines and contrary to 2H7 the two other sdAbs contain lysines in the heme recognition region, *i.e.* in CDR1 sequence and

consequently biotinylation could interfere with their binding to heme (**Chapter 2 Fig. 1F**). As a coating antibody, we choose sdAb 1A6 instead of 2H10 even though 2H10 is the most specific against “free heme”. This choice was based on the heme binding properties of the sdAbs. The sdAbs 2H7 requires Fe for heme recognition similarly to sdAb 2H10, suggesting that both recognize the same heme epitope, namely the iron while the sdAb 1A6 does not. Therefore, while the sdAb 2H7 and 1A6 do not share the same heme epitope, the same is not applied for sdAb 2H7 and 2H10, and consequently the combination of both sdAb with similar heme binding properties most probably would interfered with each other heme binding in the ELISA assay (**Chapter 2 Fig. 2; Table 1**). This is consistent with the observation that the combinations 2H10/2H7-biotin provides a lower resolution for heme calibration curve in comparison to 1A6/2H7-biotin (***data not shown***). Altogether, this justifies the use of the sdAbs 1A6/2H7 based sandwich ELISA, with a linear range between 0.15-2.5 µM (**Chapter 2 Suppl. Fig. 3**).

We then addressed the applicability of this method for quantification of “free heme” in plasma samples from healthy and hemolytic conditions. To address this question we used an experimental model in which acute hemolysis was induced in mice by phenylhydrazine (PHZ) administration (Itano et al., 1975). This drug induces hemolysis by promoting ROS production with intensive oxidative damage of RBC proteins. Moreover, PHZ promotes the formation of methemoglobin and consequently the release of heme group which accumulates in the plasma (Valenzuela et al., 1977;Berger, 2007). There are however, several caveats related to the use of PHZ to induce hemolysis, mainly due to a lack of knowledge about its effects in other cell types as well as possible side effect not related with hemolysis (Berger, 2007).

The sandwich ELISA developed to measure “free heme” was combined to other methods, to further characterize and understand the role of heme during hemolytic conditions. These include total heme measurement, heme biovailability and the heme binding capacity of plasma. As some of these methods were developed for this work, we will briefly discuss their application and then discuss the

results obtained using those methods in the previously described hemolytic mice model.

Along the years, several methods, including formic acid assay (Kuross et al., 1988;Regan et al., 2001), pyridine hemochromogen assay (Berry and Trumpower, 1987) and fluorescence assay based on oxalic acid (Vinchi et al., 2013) were used for total heme quantification*, i.e.* heme bound or not bound to any other molecule. Taking in consideration those methods, we decided to use formic acid assay, although there is no consensus about the best and most appropriate technique to be used for this purpose. The formic acid assay has the main advantages of being easy and fast to perform without the requirement of special equipment or hazardous chemicals. Importantly, it allows to measure high number (>96) of samples simultaneously and independently of the sample type (*e.g.* plasma, tissues, cells). In contrast to this, the fluorescence assay requires the use of potentially dangerous chemicals but also the sensitivity of the assay is dependent of the sample/tissue (Shaklai et al., 1985). Moreover, porphyrins, including ZnPP and PP or other molecules that are fluorescent at the same wavelength, interfere with total heme measurement (Shaklai et al., 1985). The pyridine hemochromogen assay, similarly to the fluorescent assay, requires the usage of potentially dangerous chemicals and its sensitivity depends on the volume, sample heme concentration and the presence of lipids and/or photosynthetic pigments that can interfere with the measurements (Wilks, 2002). The usage of formic acid assay was further supported by the observation that the results obtained by this method were comparable with the ones determined by other methods (Spiller et al., 2011), including the fluorescence assay (Vinchi et al., 2013) for the same type of sample.

To measure heme bioavailability, *i.e.* extracellular heme uptake by cells, as readout of heme accumulation in the cells that might be correlated with intracellular heme cytotoxicity, we adapted a previous assay referred as horseradish peroxidase (HRP) activity assay (White et al., 2013). In this assay, the reporter cells express HRP exclusively in the Golgi, that becomes active once “free heme” is provided and allows to establish when intracellular heme increases, which is directly proportional to HRP enzymatic activity. To assure that the measurement depends exclusively on

exogenous heme, it is required to supplement the cells with succinylacetone, a potent competitive inhibitor of ALA dehydratase, the enzyme of heme biosynthesis (**Chapter 1, section 2**).

The heme binding capacity assay was developed by adapting the previous sdAbs-based sandwich ELISA, to determine the heme binding/buffer capacity of plasma. The heme binding capacity is defined as the concentration of "free heme" required to reach half-saturation of the total heme binding capacity of plasma. Heme binding capacity is revealed when by providing increasing concentrations of heme to the plasma, presumably mimicking hemolytic conditions in which cell-free hemoglobin releases heme and allows it to bind to certain components of the plasma for a certain period of time. Once the heme binding capacity of plasma is saturated the excess "free heme" becomes available and is bound by sdAb-sandwich based ELISA. The heme binding capacity of plasma is presumably provided by proteins, lipids or other molecules with an affinity higher towards heme, thus avoiding the detection of "free heme" by the sdAbs used in the ELISA. This newly developed assay should allow for the first time to assess whether under pathological conditions associated with hemolysis, the buffer capacity of the plasma is changed and test whether this might be correlated with the severity of the disease.

Despite the accumulation of total heme in plasma during acute hemolysis induced by PHZ (**Chapter 2 Fig. 7A**), "free heme" remained undetectable (lower than 0.15 µM), as measured by the sandwich-based ELISA (**Chapter 2 Suppl. Fig. 3**). This result contradicts previous studies that described an increase of "free heme" associated with the pathology of hemolytic diseases (Pamplona et al., 2007;Ferreira et al., 2008;Seixas et al., 2009;Larsen et al., 2010;Gozzelino et al., 2012). As previously mentioned, this might be associated with limitations on the methodologies used for this purpose. Nonetheless, it was observed an increase in total heme as well as heme bioavailability during acute hemolysis (**Chapter 2 Fig. 7A and B**) which is correlated with a decrease in the heme binding capacity of the plasma (**Chapter 2 Fig. 7C**). This is consistent with the notion that acute hemolysis, is associated with heme bioavailability which presumably leads to cellular heme

toxicity. Despite a transient decrease in plasma heme binding capacity, heme released from cell free hemoglobin is most probably always bound to plasma compound(s) with an affinity superior to 10^{-7} M, hence not detectable by the sdAbs based ELISA. This transient decrease in the heme binding capacity of plasma is followed by a rapid recovery to steady state (**Chapter 2 Fig. 7C**). Taking into consideration these results, we propose the existence of a tight regulatory mechanism for restoring systemic heme homeostasis. This mechanism is most probably mediated by heme scavenger proteins that by avoiding the generation of "free heme" inhibiting its cytotoxic effect. Most probably hemopexin do not participate in this mechanism, given that deletion of the hemopexin gene has no effect on the overall plasma heme binding capacity in mice (***data not shown***). Therefore, other heme binding proteins, such as albumin, might participate in this mechanism.

Another important observation is that under homeostatic conditions, the heme binding capacity of the plasma is very high (10^4µM range) in comparison with total heme measured (10^1µM range). This further supports the existence of highly controlled regulatory mechanisms for maintenance of heme homeostasis that does not allow the accumulation of heme in the plasma as a free molecule. However, in very preliminary experiments, we observed "free heme" accumulation in urine of hemolytic mice, namely in the DBA/2 mouse strain infected with *P. chabaudi chabaudi,* at day seven after infection (parasitimia >60%). The concentration of "free heme" in these samples was about 3 µM (*data not shown*) suggesting that, although no "free heme" is detected in plasma, there is a mechanism of heme detoxification through the urinary tract. Therefore, the heme released from cell-free hemoglobin is not only eliminated by catabolic degradation by HO-1 (**Chapter 1 section 5.1**) (Gozzelino et al., 2010), but presumably by its transport and excretion in the urinary tract This hints to a new mechanism for the maintenance of systemic heme homeostasis, preventing not only the presence of "free heme" in plasma but also its prolonged binding to heme scavengers. The undesirable binding of heme to some of its scavengers might be correlated to a mechanism to prevent the saturation of its scavenger and/or to avoid heme retention by them which might lead

to an increase or a low inhibition in heme reactivity and thus not preventing its deleterious effect. This hypothesis is supported by the observation that the peroxidase activity of heme is partially (50-60%) inhibited by albumin while greatly suppressed (80-90%) by hemopexin (Grinberg et al., 1999).

We cannot exclude other possibilities for detecting heme in the urinary excretion, such as kidney damage which might lead to the release and accumulation of proteins (*i.e.* proteinuria), including hemoglobin that would release heme upon unfolding due to the high urea concentration, a natural inducer of protein denaturation (Hua et al., 2008). Another possibility is the presence of a specialized mechanism for heme release through the urinary track. Nevertheless, further studies are required to confirm these results and understand further the mechanism involved in this process. Future experiments should allow to understand whether “free heme” is generated by cell-free hemoglobin in the plasma or by the accumulation of hemoglobin in urine where the heme is released.

In the present work, we have shown that upon hemolysis no “free heme” is detected in plasma but a small portion of heme is uptaken by cells, presumably leading to heme driven cytotoxity (Larsen et al., 2010;Gozzelino and Soares, 2011;2014). Heme acts as pro-oxidant molecule (**Chapter 1 section 6.1**), leading to the formation of ROS, an effect that might justify the sensitization of cells to TNF mediated programmed cell death (Gozzelino et al., 2010;Larsen et al., 2010;Gozzelino et al., 2012;Larsen et al., 2012). This notion is further supported by the observation that heme bound to certain lipids and proteins from cell membrane as well as lipoproteins, such as LDL, cause their oxidation with the production of highly toxic substances, such as lipid peroxidase, F2-isoprostanes (Balla et al., 1991;Camejo et al., 1998;Jeney et al., 2002). For that reason, we assessed whether the sdAbs developed during this work would prevent the pro-oxidant effect of heme when released from hemoglobin. We performed an *in vitro* assay to verify the biological effects of the sdAbs once bound to heme. Indeed, sdAb 2H10 inhibits the pro-oxidant activity of heme (**Chapter 2, Fig. 8A and B**), as assessed by an ascorbate oxidation based assay. This inhibitory effect is even more efficient than the plasma heme scavenger albumin, at least on a mol-per-mol basis (**Chapter 2**

Fig. 8A and B). As previously mentioned, the anti-oxidant effect of sdAb might be particularly useful when envisioning the use of anti-heme sdAb as a therapeutic approach to target the pro-oxidant effects of extracellular and soluble “free heme”, presumably preventing its pathogenic effects. Besides that, this sdAb targets heme specifically, *i.e.* not contained in hemoproteins, avoiding a possible toxic effect upon sdAbs administration. Therefore, this approach should be less toxic when compared to others than might affect heme function in those hemoproteins (Pamplona et al., 2007;Gozzelino et al., 2010). Further tests *in vivo*, such as the sdAb treatment of mice models of immune mediated inflammatory disease associated with hemolysis, are needed to confirm whether this strategy is viable.

We asked whether the anti-oxidant effect provided by the sdAbs could be complemented by inhibition of heme cellular uptake. The sdAbs 2H10 is inefficient to prevent heme cellular uptake, consistent with its inability to prevent heme sensitization to programmed cell death (Larsen et al., 2010;Gozzelino and Soares, 2011) (**Chapter 2 Fig. 8D**). This contrasts with the high affinity heme scavenger hemopexin, which suppresses heme cellular uptake (**Chapter 2 Fig. 8D**) as well as heme driven cytotoxicity (**Chapter 2 Fig. 8C**). This may be explained by the higher affinity of cellular heme importers towards heme, *e.g.* HRG-1, HCP-1 or FLVCR2 (Duffy et al., 2010;Le Blanc et al., 2012;White et al., 2013), as compared to the sdAb but not as compared to hemopexin. If this was to be the case, then heme may be transfer/deliver by the sdAb to these heme transporters. And as such these sdAbs might work as anti-oxidant soluble heme transporters rather that as heme scavengers, allowing for heme delivery to the cells and degradation by HO-1 (**Chapter 1 Fig. 7**).

In a future work, the sdAb we developed will be further improved, using standard procedures to increase affinity towards their cognate ligand (Barbas, 2001). This would allow the generation of specific anti-heme sdAbs with different affinities and as such with the ability to remove heme from distinct compounds to which heme binds upon release from hemoproteins. For instance, heme binding to lipoproteins, such as LDL, promotes lipid peroxidation with the production of highly deleterious products (Balla et al., 1991;Jeney et al., 2002). Generation of sdAbs

with an affinity towards heme higher than LDL should avoid this effect and may therefore have beneficial effects in countering pathologic conditions in which LDL oxidation acts in a pathogenic manner. The same reasoning can be applied to other cognate interactions between heme and proteins and/or lipids in plasma. It is probably not desirable to develop sdAbs that bind heme with an affinity higher than the one of hemopexin since the removal of heme from this physiologic heme scavenger might have undesirable biological effects.

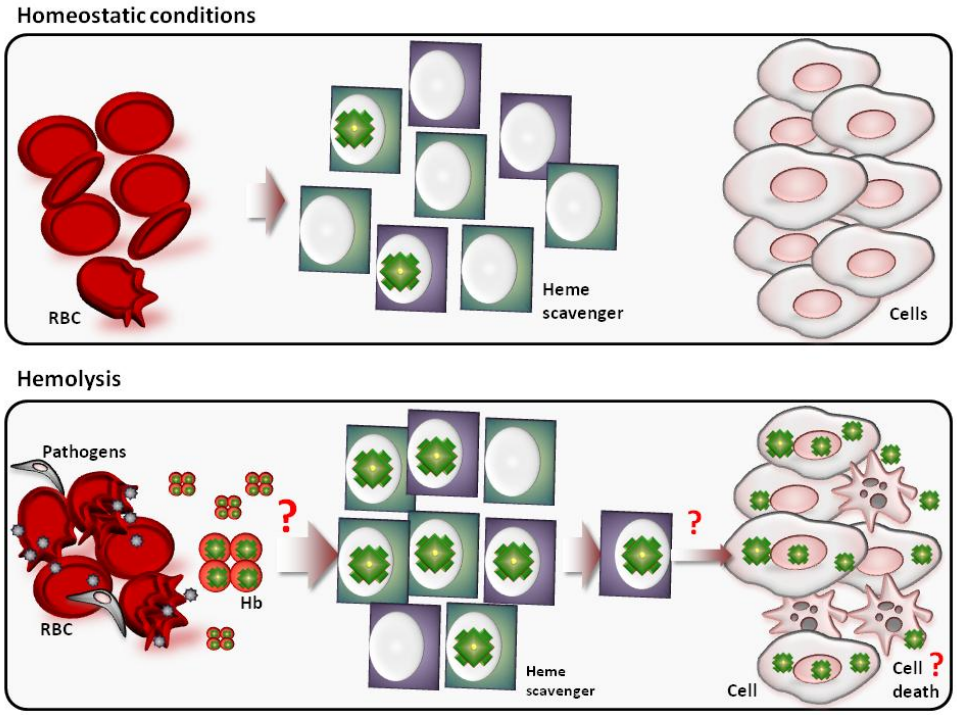


Figure 2: Model of heme distribution upon hemolysis according to the results obtained in this work. In homeostatic conditions, the plasma has a very high heme binding capacity (HBC) provided by proteins/lipids or other molecules with heme scavenger capacity. Upon hemolysis, cell free-hemoglobin releases its heme that is scavenged/bound by those heme scavengers, decreasing the HBC of the plasma. Besides that, some of the heme scavengers presumably transfer heme to the cells leading to an increase in intracellular heme that might be cytotoxic.

In supplementary information, a preliminary work was developed to generate constructs based on the developed sdAbs, namely sdAbs 1A6 and 2H7. These constructs were obtained by incorporation of a CDR3 region into the sdAbs as well

as by dimerization of the sdAbs (**Supplementary Information Fig 1**). Therefore, generating constructs with higher binding avidity (number of heme binding sites per sdAb) and with monospecificity or bispecificity characteristics, which might be therapeutically desirable (**Supplementary Information**).

In conclusion (**Fig. 2**), this study reveals that anti-heme sdAbs can be used not only to detect cellular heme but also to quantify “free heme” in biological samples. In this thesis we also showed using a newly developed sdAbs-based assay coupled with other methodologies that, under hemolytic conditions, there is an increase of total heme associated with a cellular heme accumulation and a decrease of heme binding capacity in the plasma, which is rapidly recovered to steady state conditions. This heme accumulation in plasma and cells might act in a deleterious manner, contributing to the pathogenesis of the disease associated with hemolysis, Furthermore, we showed that anti-heme sdAb can modulate heme biologic activity, namely its pro-oxidant effect which might be therapeutically relevant.

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APPENDIX

SUPPLEMENTARY INFORMATION

Engineering of sdAbs: Dimerization of sdAbs against heme and generation of sdAbs containing CDR3

Engineering of sdAbs: Dimerization of sdAbs against heme and generation of sdAbs containing CDR3

Zélia Gouveia[1,6], Frederico Aires-da-Silva[2,5], João Gonçalves[3,4] & Miguel P. Soares[1,a*]

[1]Instituto Gulbenkian da Ciência, 2780-156 Oeiras, Portugal.; [2]Technophage S.A. 1600-190 Lisboa, Portugal.; [3]CPM-URIA Faculdade Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal.; [4]IMM, Faculdade. Medicina, Universidade de Lisboa, 1649-028 Lisboa, Portugal.; [5]CIISA-Faculdade de Medicina Veterinária, Universidade de Lisboa, 1300-477 Lisboa, Portugal. [6]Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa

*To whom the correspondence should be addressed: Miguel P. Soares; Inflammation Group, Instituto Gulbenkian de Ciência, Rua da Quinta Grande, 6, 2780-156 Oeiras, Portugal, Tel.: +351-214464520; E-mail: mpsoares@igc.gulbenkian.pt

Keywords

Antibodies, Heme, sdAbs constructs, Complement determinant region 3 (CDR3), antibody engineering, single domain antibodies

Author contribution

ZG designed, performed and analyzed all the experimental work. JG and FAS conceived and designed the synthetic phage display library and contributed critically to screening of sdAbs by Phage display technology. MPS designed and supervised the study and wrote the manuscript with ZG

PRELIMINARY RESULTS

1. ABSTRACT

Single domain antibodies (sdAbs) can target biologic compounds involved in a variety of pathologic conditions. However, their small size might compromise antigen functional affinity, due to a decrease in avidity. To overcome this limitation, sdAbs can be engineered to combine two domains, such as variable light chain (VL)-VL domains through a flexible linker thus increasing their avidity (and molecular weight) and maybe stability and their intrinsic affinity, which might be desirable in certain therapies. In this work, we show preliminary results of engineered sdAbs into a multidomain antibody (dimer) as well as sdAbs with a CDR3 recognizing heme region, based on the previously described sdAb 1A6 and 2H7 developed against “free heme” (Chapter 2). Moreover, the engineered sdAbs were developed to be monospecific or bispecific, which might improve heme recognition. Their constant binding affinity for heme (K_D=10^{-7}M) is similar to the parental sdAbs, but with an increased avidity, as expected. Amongst the engineered sdAbs, the specificity for heme was tested for CDR1-CDR3 2H7-2H7, revealing a decrease in specificity, as compared to parental sdAb 2H7, in that this sdAb acquired the capacity to recognize free heme without its iron. Further studies are required to better characterize heme binding by all sdAbs constructs as well as their possible applications.

2. INTRODUCTION

Engineering of antibodies to low weight functional antibodies fragment (~15 kDa), also known as single domain antibodies (sdAbs), offers several advantages in comparison to higher molecular weight conventional antibodies (150-160 kDa) or other recombinant antibody fragments (**Chapter 2**) (Gronwall and Stahl, 2009). These include high stability independently of the CDR variability, high expression levels or increased solubility and capacity of tissue penetration *in vivo*. Besides that, they are easy and low cost to produce and purify (da Silva et al., 2008). These characteristics make them suitable for different biotechnological and therapeutic applications. However, decreasing their size to the minimum possible also decreases their antigen recognition site, *i.e.* CDRs, eventually compromising

antigen functional affinity, also referred as binding avidity. This limitation can be overcome by protein engineering and recombinant DNA technology used for sdAb assembling into stable multimeric antibodies with high binding avidity and specificity (Holliger and Hudson, 2005;da Silva et al., 2008). These include the generation of single chain variable fragments (scFv) usually with two variable domains, light (VL) and heavy (VH), joined by a flexible polypeptide linker composed by serines and glycines (Bird et al., 1988;Kortt et al., 2001;Simmons et al., 2006;Gronwall and Stahl, 2009;da Silva et al., 2012). The scFv has typically a molecular weight of 30 kDa and a higher antigen functional affinity provided by multiple antigen binding sites, *i.e.* increased avidity. Generally, these type of antibodies are designed to be monospecifc, however, they can also be engineered to be bispecific, recognizing two different adjacent epitopes in the same antigen which improves their avidity (da Silva et al., 2008;Glaven et al., 2012). Alternatively, they can cross-link two different antigens which might be advantageous in therapeutic applications (da Silva et al., 2008;da Silva et al., 2012). In summary, by antibody engineering it is possible to fine-tune diverse antibodies features, such as avidity, valency, stability, molecular weight and their intrinsic affinity, which all might be desirable therapeutically.

In this study a panel of constructs were developed based on the previously described sdAb 1A6 and 2H7 generated to target “free heme”, a highly reactive molecule that contributes critically to the pathogenesis of hemolytic conditions, as demonstrated for sickle cell disease (Belcher et al., 2014), malaria (Pamplona et al., 2007;Ferreira et al., 2011a) or severe sepsis (Gozzelino et al., 2010;Larsen et al., 2010) (**Chapter 2**). These include the generation sdAb multidomains, namely dimers with a similar size of the scFv and the incorporation of a CDR3 region into these sdAbs composed by the amino acids from the CDR1 region of the sdAb 1A6 and 2H7.

3. RESULTS

3.1 Generation of different constructs of based on previously described sdAbs

Several constructs based on sdAbs 1A6 and 2H7 (**Chapter 2**) were designed simultaneously. These include, four single-chain variable fragments (sc-Fv) generated by dimerization of the sdAbs 1A6 and 2H7 through a linker fragment composed by glycines (G) and serines (S) in a total 18 amino acids; S G G G G S G G G G S G G G G S S G. Other four construct were developed to incorporate into the structure of sdAbs 1A6 and 2H7 a CDR3 region that corresponds to the previously determined CDR1 sequence of the sdAbs 1A6 and 2H7 (**Fig. 1**).

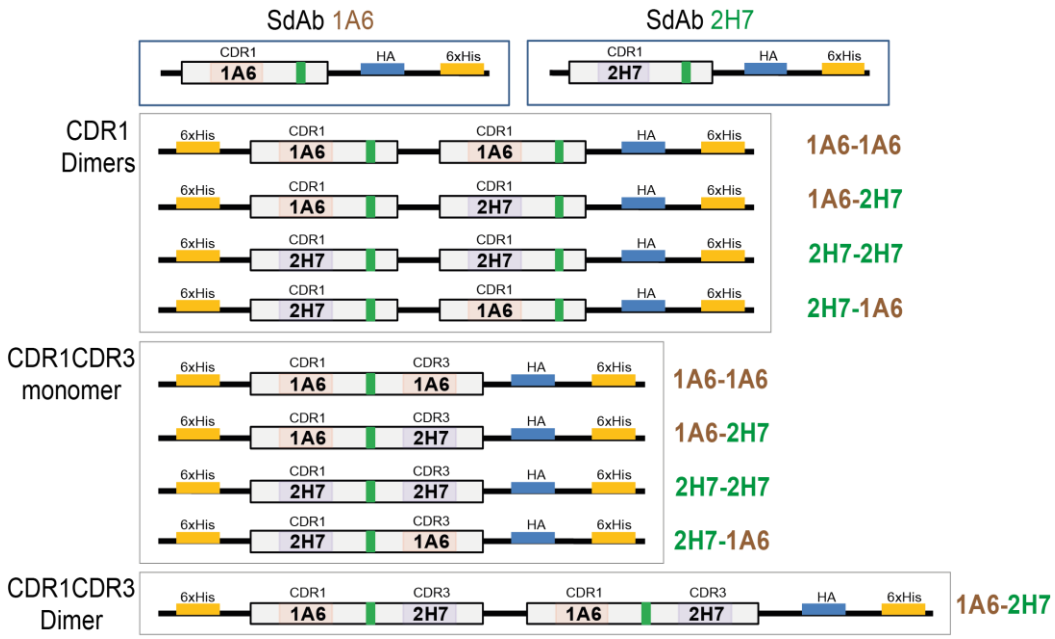


FIGURE 1. Generation of sdAbs constructs. The sdAbs constructs were designed based on the sequences of the sdAbs, 2H7 and 1A6 raised against heme (**Chapter 2**). The CDR1 dimers, also referred as scFv were constructed by overlapping PCR of the two sdAb sequences, covalently linked with a long peptide linker of 18 aminoacids (SGGGGSGGGGSGGGGSSG). The monomers CDR1CDR3 were synthesized by NZYTech® as well as the CDR1CDR3 dimers.

Finally, one scFv was generated from the dimerization of two sdAbs containing CDR3 region, namely sdAb CDR1CDR3 1A6-2H7.

3.2 Constructs expression in different bacteria strains and their purification

To improve the expression of the different constructs in a bacterial expression system, we used different bacterial strains of *E. coli* known to provide optimized conditions for protein expression (**Fig. 2**). These include the bacterial strains “BL21 codon plus” that provides tRNAs rarely used in *E. coli* , “Tuner” and “Shuffle”, the latter being engineered to provide favorable conditions in the bacteria cytoplasm for disulfide bond formation of proteins. The ratio between the protein expressed upon induction compared to its base line reveals that between all the constructs tested, including the parental sdAb 1A6 and 2H7, the best is obtained for the “Shuffle” strain. Therefore, the protein expression for protein purification was performed in this bacterial strain (**Fig. 2A**). The construct expression in larger scale (1 liter) shown a higher expression of the protein, however restrained to the inclusion bodies fraction (*data not shown*). For that reason a denaturating protocol for protein purification was used followed by the re-folding using a method for fast removal of the denaturing buffer to non-denaturing (**Fig. 2B**). This method led to higher percentage of protein precipitation (>90%), however for most of the construct it was possible to recover protein aliquots with a concentration of around 1 mg/mL, while for CDR1CDR3 2H7-2H7 construct was aliquots of 3 mg/mL (*data not shown*). The issue related with protein precipitation can be overcome by using a step-wise dialysis, as shown for the parental sdAbs (**Chapter 2**). Furthermore, the purification protocol used leads to protein purity of about 90% as assessed by SDS-PAGE (**Fig. 2B**). This assay also confirms the molecular size of CDR1CDR3 monomers of about 20kDa, an increase of about 3kDa in comparison to the parental sdAbs 1A6 and 2H7 matching expected size of the incorporated CDR3 region (**Fig. 2B**). The CDR3 dimer is approximately 40 kDa as it was generated by the combination of two CDR1CDR3 monomers (20kDa) while the CDR1 dimers are approximately 35 kDa as they were generated from the parental sdAb (17kDa) (**Fig. 2B**).

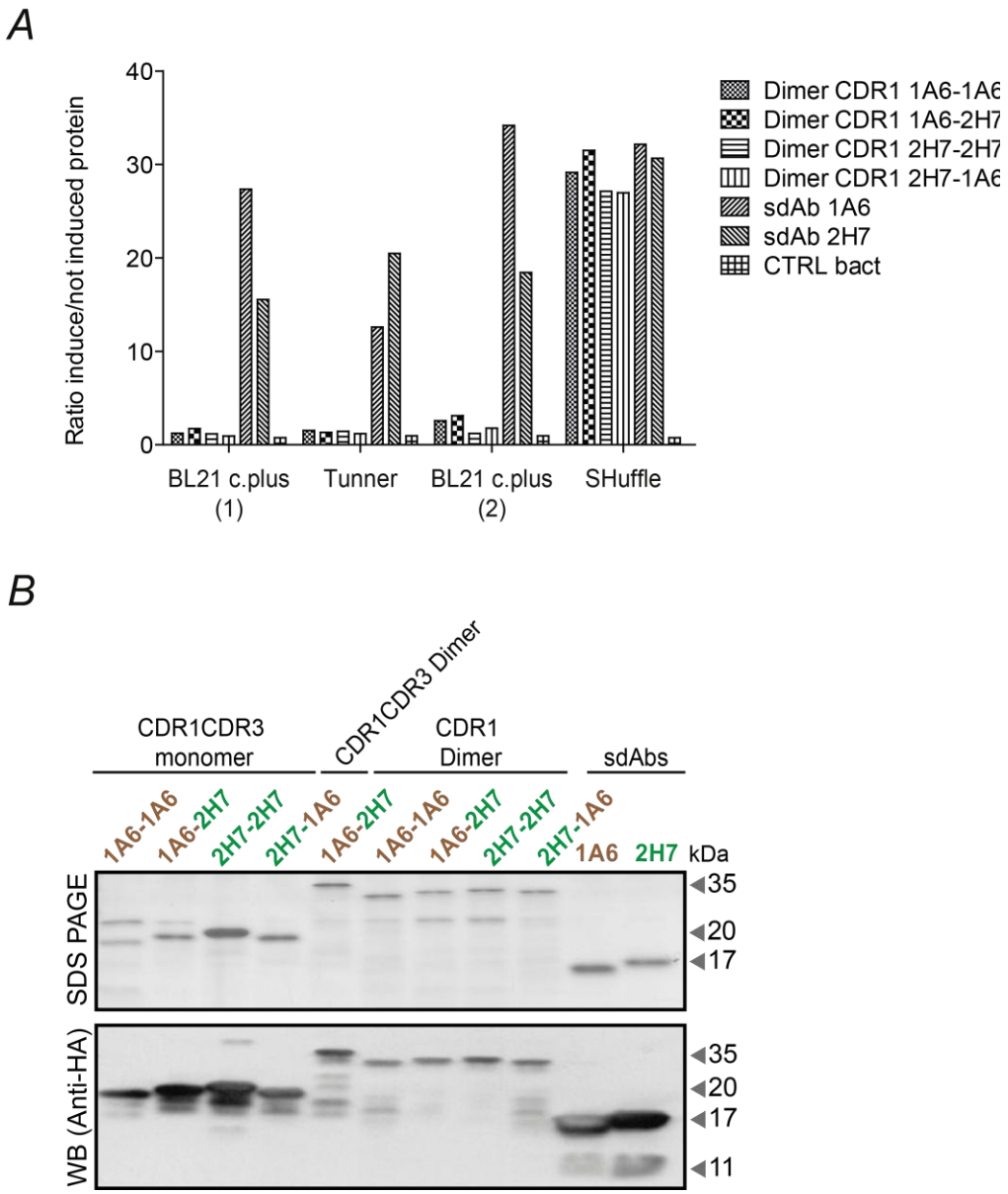


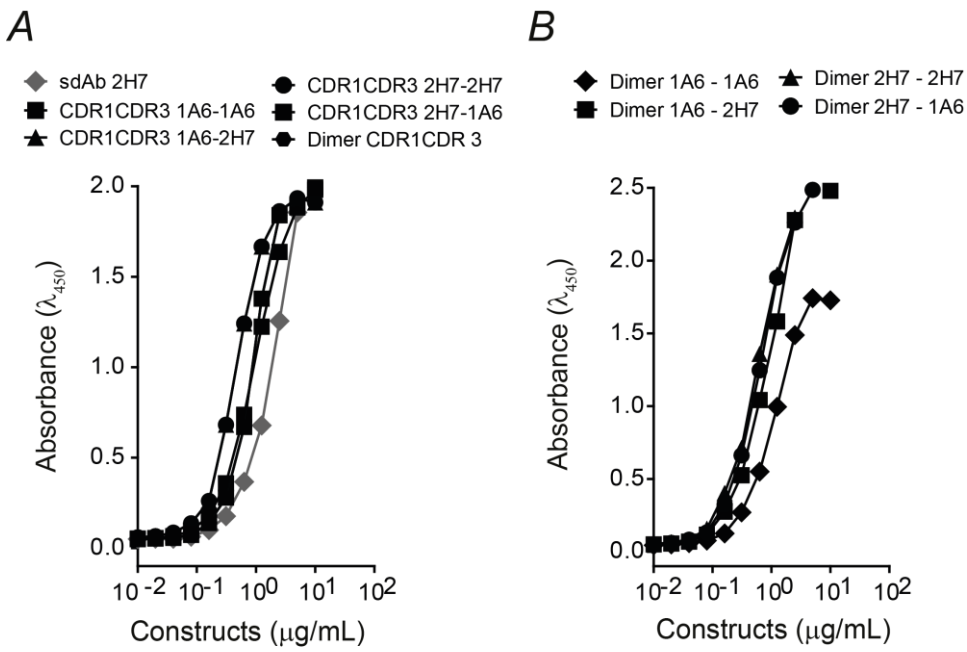
FIGURE 2. Protein expression in different bacteria strains and their purification. (A) The ratio between the protein expression upon induction in comparison to the same protein in non inducing conditions using the bacterial strains BL21codon plus, tunner and Shuffle. The constructs expressed were the dimers and the parental sdAb 1A6 and 2H7. (B) SDS-PAGE of purified constructs detected by coomassie-based stain or by western blot using an anti-HA mAb.

In the western blot, the lower bands observed for some of the constructs might be due to some extent to protein degradation and to the expression of a ~17kDa sdAb without the ~2kDa peptide leader (MKKTAIAIAVALAGFATV), similarly to the parental sdAbs (**Fig. 2B**) (**Chapter 2 Fig. 1D**).

3.3 Heme Binding

The binding of these engineered proteins were assessed by ELISA using an ELISA plate coated with heme, showing that all of them can bind heme in a similar manner that the parental sdAb 2H7 with exception of the dimer 1A6-1A6 (**Fig. 3A, B**). These results were confirmed by surface plasmon resonance (SPR; BIAcore system) (**Fig. 3D**) and their affinity constant (K_D) for heme determined (**Fig. 3C**). The K_D of the different constructs are around 10^{-7} M, similar to what was obtained for the parental sdAb 1A6 and 2H7. The 2H7-2H7 and 2H7-1A6 dimers as well as the CDR1CDR3 1A6-1A6 show a drop in K_D to 10^{-6} M (**Fig. 3C**). The kinetic of heme binding to construct CDR1CDR3 2H7-2H7, which is representative of all the others (**Fig. 3D**), revealed a fast on-rate (association, 100 to 300s) for heme followed by a considerable fast off-rate (dissociation, 300 to 900s), similarly to the parental sdAbs. These results suggest that while the constructs can scavenge rapidly heme, they are also prone to release it.

Although the heme binding affinity of the constructs does not increase compared to the parental sdAbs, they display a higher functional affinity, also known as avidity, that is provided by their multiple heme binding sites.



C

	SdAb	K_D(nM)
CDR1 Dimers	1A6-1A6	660
	1A6-2H7	876
	2H7-2H7	2060
	2H7-1A6	1060
CDR1CDR3 monomer	1A6-1A6	1060
	1A6-2H7	252
	2H7-2H7	**468**
	2H7-1A6	372
CDR1CDR3 Dimer	1A6-2H7	638

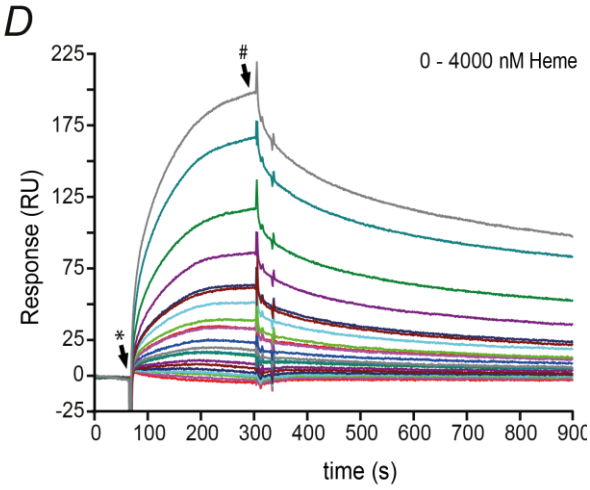


FIGURE 3. Binding to heme (A) Relative binding of CDR1CDR3 monomers and dimers to heme detected by ELISA in comparison to the parental sdAb 2H7. (B) Relative binding of dimer to heme detected by ELISA. (C) Binding affinity of construct towards heme, determined by BIAcore surface Plasmon resonance. (D) Sensogram of heme binding to CDR1CDR3 2H7-2H7. For the determination association and dissociation kinetic rate constants, used to calculate the constant of affinity (K_D) from the sdAb was immobilized in a CM5 chip using amine coupling at ~800 resonance units (RU) and different concentration of heme (from 0 to 4000 nM) injected for 4 min. The symbol * indicates the start and # the end of heme injection.

3.4 Specificity of the sdAbs constructs

The specificity towards heme was determined for one of the constructs, the CDR1CDR3 2H7-2H7 (**Fig. 5**). The specificity for the other constructs will be determined in future work. For the determination of specificity, a previously developed ELISA (**chapter 2, section 3.2**) was used, in which the sdAbs CDR1CDR3 2H7-2H7 was pre-incubated with heme (Iron protoporphyrin; FePP), PP IX (PP; the metal-free precursor of heme), zinc PP (ZnPP, produced physiologically by ferrochelatase during iron deficiency), cobalt PP (CoPP, contain a Co instead of Fe), gallium protoporphyrin (PP) IX (GaPP; a non-iron metalled porphyrin containing a similar sized but redox inert gallium atom) or tin PP (SnPP, containing Sn instead of Fe) (**Fig. 4**).

Pre-incubation of the CDR1CDR3 2H7-2H7 with all the heme analogs inhibit its binding to heme (**Fig. 5A**), while the sdAb 2H7, previously described does not recognize PP (**chapter 2, Fig. 2**). Apparently, the insertion of the CDR3 region modify its specify when compared with the sdAb 2H7. These results suggest that the central metal is not essential for heme recognition as well as Fe redox state as shown by the recognition of GaPP.

To verify the influence of porphyrin side chains in heme recognition, the CDR1CDR3 2H7-2H7 was pre-incubated with Fe mesoporphyrin IX (MesoP) or Fe deuteroporphyrin IX (DeutP), synthetic heme derivatives in which the vinyl group was protonated or removed from heme, respectively (**Fig. 4**). The Fe tetraphenylporphyrin (FeTPP), a synthetic heterocyclic molecule that resemble heme (**Fig. 4**) was also tested. The synthetic heme derivates were recognized by the constructed with exception of the FeTPP, suggesting that heme recognition involves a carboxylic acid of one of the two propionate side chains or that the presence of the phenyl groups might inhibit its recognition (**Fig. 5B**). To access whether the propionate group of heme are essential for heme recognition by this CDR1CDR3 2H7-2H7, the heme analog FePPCH3 should be used, as in **Chapter 2 section 3.1**. The recognition of biliverdin and bilirubin, two end-products of heme degradation by heme oxygenase-1 and biliverdin reductases, respectively, were tested. The CDR1CDR3 2H7-2H7 fails to recognize both (**Fig. 5C**).

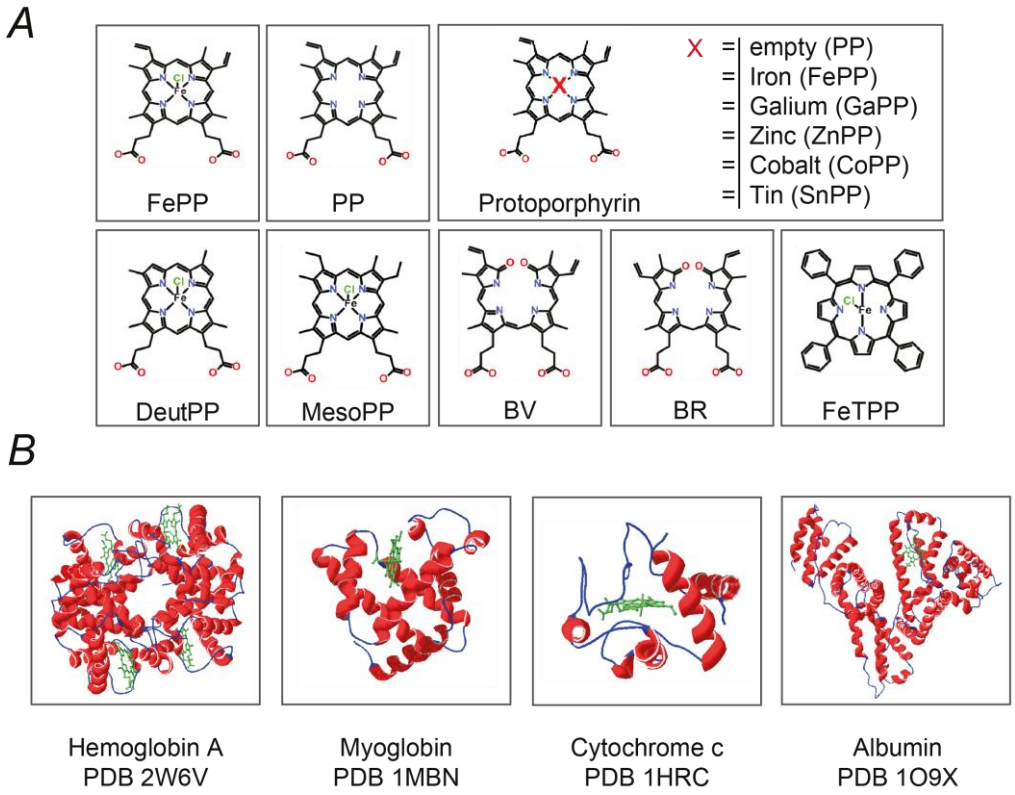


Figure 4. Structure of the different heme analogs (A) Heme (FePP, ChEBI: 50385) and heme analogs PP (ChEBI: modified 50385), ZnPP, CoPP, GaPP and SnPP as well as MesoP (ChEBI: 50385), DeutP (ChEBI: modified 50385) and FeTPP(CAS mol: 16456-81-8). The heme degradation end product biliverdin (BV, ChEBI: 17033) and bilirubin (BR, ChEBI: 16990) are also represented (B) Crystalographic structure of Hemoglobin, myoglobin,, Cytochrome c and human serum albumin bound to heme (HSAH) and their respective protein data base (PDB) code .

To test whether the CDR1CDR3 2H7-2H7 can recognize heme when incorporated in proteins, it was pre-incubated with Hemoglobin, oxidized hemoglobin (MetHb), myoglobin (Mb), albumin containing heme *b* or cytochrome c, which demonstrated that it failed to recognize heme bound to proteins (**Fig. 5D**). This suggests that, similarly to the sdAb 2H7 that was used to generate this construct, it does not recognize tightly bound heme, as present in hemoproteins.

We cannot exclude however, that this CDR1CDR3 2H7-2H7 might recognize heme once bound to other proteins.

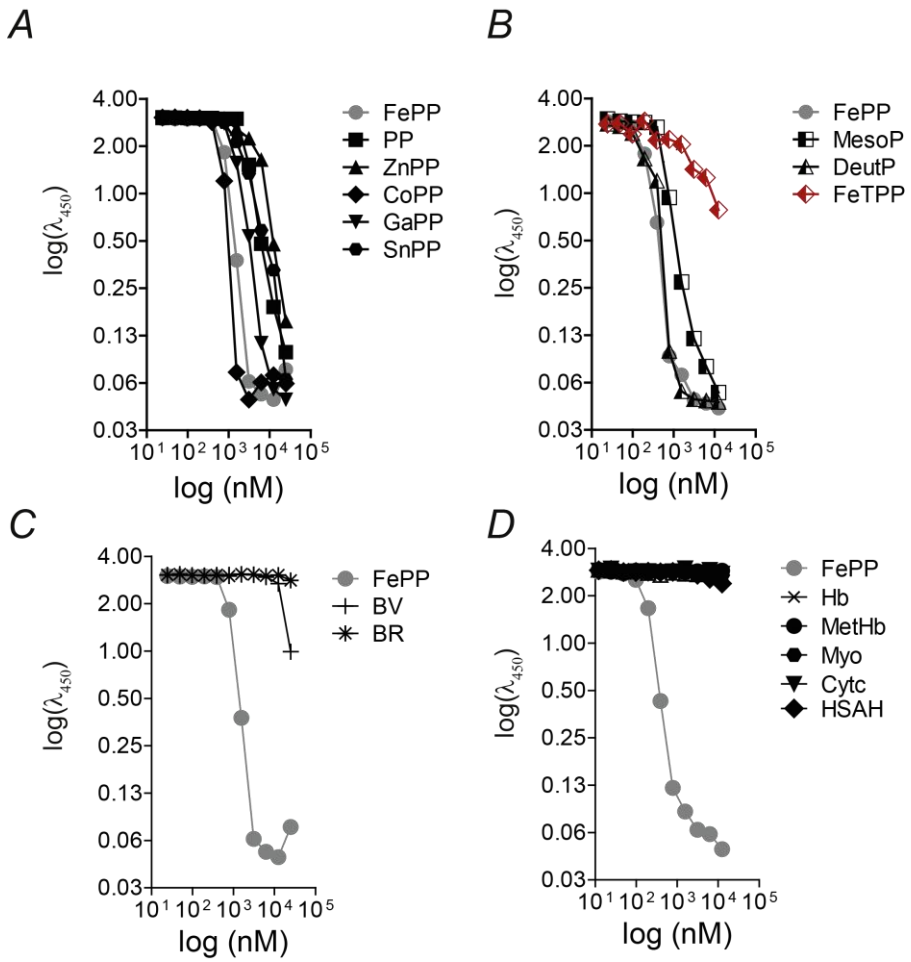


Figure 5. CDR1CDR3 2H7-2H7 heme binding specificity to heme, its analogs and hemoproteins. (A-D) Increasing concentrations of different compounds were tested for their ability to interfere with CDR1CDR3 2H72H7 binding to solid phase bound heme, as measured by the ELISA. (A) PP, ZnPP, CoPP, GaPP and SnPP. (B) MesoP, DeutP, FeTPP(C) Hb, oxidized Hb (MetHb), Mb, Cytochrome c (Cytc) and human serum albumin bound to heme (HSAH). Hemin (FePP) was used as a positive control. Data shown is representative of three independent experiments.

4. DISCUSSION

SAbs targeting specifically “free heme” were developed and characterized for their specificity for heme and heme-sdAbs binding, using a combination of different biochemical techniques (UV-visible, Resonance Raman, Circular Dichroism and Fourier transform infrared spectroscopy), revealing that sdAbs bind specifically to “free heme” (**Chapter 2**). When released from hemoproteins heme acts as a pathogenic compound and becomes vasoactive (Belcher et al., 2014), pro-inflammatory(Graca-Souza et al., 2002;Figueiredo et al., 2007) and cytotoxic(Seixas et al., 2009;Gozzelino et al., 2010;Larsen et al., 2010), therefore promoting the pathogenesis of hemolytic diseases such as sickle cell disease(Ghosh et al., 2013;Vinchi et al., 2013;Belcher et al., 2014), malaria(Pamplona et al., 2007;Ferreira et al., 2011b) or severe sepsis(Larsen et al., 2010). These sdAbs were then used to developed bioassays allowing quantitative analyzes of “free heme” in biologic samples as well as the detection of cellular “free heme”. We also demonstrated that these sdAbs can be used to suppress the labile redox activity of “free heme”, which may be relevant when targeting it therapeutically (**Chapter 2**). In the present work, antibody engineering was used to developed new constructs based on previously described sdAbs 1A6 and 2H7, although the sdAb 2H10 shown to be the most specific against heme (**Chapter 2**). Thus future work should aim at developing new constructs based on this sdAb and assess their biologic function. We expect these new constructs to display high binding avidity for heme, presumably important for heme-specific targeting. Besides that, the stability and expression of these sdAb might be significantly improved. In this preliminary work, the several constructs were generated by dimerization of the sdAbs 1A6 and 2H7 as well as by the incorporation of a CDR3 region into the original sdAbs 1A6 and 2H7 (**Fig. 1)**.

The incorporation of the individual sdAbs into a dimeric format produced a protein with approximately 30 kDa, similar to a scFv. These constructs were designed to be monospecific, such as dimers 1A6-1A6 or 2H7-2H7 or bispecific, such as the dimers 1A6-2H7 or 2H7-1A6. The bispecific constructs were designed with two different order in their protein sequence, first sdAbs 1A6 followed by 2H7

and vice-versa, since it might influence the functionality of the construct. However, we did not assess this question and further studies are required (**Fig. 1**). All dimeric constructs contain a flexible glycine-serine linker joining them, allowing flexibility between the two domains which might be advantageous for their interaction with the antigen, however a higher flexibility might also promote the occlusion of the binding site to the antigen (**Fig. 1**). In this work, the linker used seems to affect heme recognition by the dimers (**Fig. 3**).

Other constructs were developed to accommodate in their structure a CDR3 sequence, similar with the CDR1 sequence of sdAbs 1A6 or 2H7, generating monospecific or bispecific monomeric sdAbs. These constructs have similar number of heme binding sites, as compared to equivalent dimers, with exception of CDR1CDR3 1A6-2H7, which has four heme binding sites (**Fig. 1**) and presumably the highest functional affinity (avidity) of all sdAbs we have generated. Whether, it is more advantageous to have a dimer of sdAb or to incorporate the CDR3 into the sdAbs remains to be elucidated.

Heme recognition by these constructs was assessed by ELISA, showing that all contructs can bind heme in a similar manner, as compare to the parental sdAb 2H7 (**Fig. 3**). The binding affinity of these constructs was determined, revealing that they have a similar affinity in the range of 10^{-7} M, as compared to the parental sdAbs (**Fig. 3**). Interestingly, sdAbs coupling did not modify their affinity, as compared to their parental constructs. However, the dimerization as well as the incorporation of the CDR3 region with ability to bind heme, presumably leads to an increase in their overall avidity. Furthermore, the kinetic curve of heme binding show a fast rate of association/dissociation, similar to the parental sdAbs (**Fig. 3D**). Although the constant binding affinity is maintained, presumably the heme binding avidity increases from one to two and to four with monospecificity/bispecificity properties, respectively. Amongst the constructs developed, CDR1-CDR3 2H7-2H7 was selected to assess their specificity for heme, based on an empirical observation in which this construct appears to be the highly stable, with high yield of protein expression/production. The specificity of other constructs should be determined in future work. The CDR1-CDR3 2H7-2H7 shown to be able to

recognize heme analogs, including DeutP but not FETPP (**Fig. 5**), suggesting that the carboxylic groups of heme are required for heme recognition. Alternatively, FETPP might be inaccessible to the construct due to a change in the protoporphyrin structure with implication in their physical/chemical properties. Similarly to the parental sdAbs, 2H7 does not recognize end-products of heme degradation by HO-1 (**Fig. 5C**), as well as heme once bound to the hemoproteins tested (**Fig. 5D**), therefore avoiding any putative cytotoxicity associated to heme binding in hemoproteins which might compromise their biologic activity. The characterization of these constructs requires further experiments (**chapter 2**), including the study of their biological effect upon heme targeting.

In conclusion, these preliminary results suggest that it is possible to engineer sdAbs into multimeric proteins without losing their ability to target heme, which might be therapeutically advantageous.

5. EXPERIMENTAL PROCEDURES

Tetrapyrroles preparation - Concentration of different tetrapyrroles (Frontiers Scientific®, Utah, USA) in solution was determined spectrophotometrically using appropriated solvents. Briefly, heme (*i.e.* hemin) stock solutions were prepared in 0.1 M NaOH, buffered with 0.1 M HCl to pH 7.4. Alternatively, hemin was diluted, as for all other tetrapyrroles, in dimethyl sulfoxide (DMSO). Hemin concentration was determined at λ_{405nm} using a extinction coefficient (E_{mM}) of 85.82, DeutP (λ_{392nm}; E_{mM}=170), MesoP (λ_{394nm}; E_{mM}=170) and GaPP (λ_{413nm}, E_{mM}=249) (43). PP (λ_{408nm}; E_{mM}=297) concentration was determined in 1.5 M HCl (43), ZnPP (λ_{415nm}; E_{mM}=150) in ethanol (43); biliverdin (λ_{377nm}; E_{mM}=51.5) in methanol (44) and bilirubin (λ_{451nm}, E_{mM}=60) in chloroform (45). CoPP, SnPP, FeTPP and CoTPP concentrations were calculated gravimetrically according to their molecular weight (MW) =654.6, 750.26, 679.99, 704.02 and 707.1, respectively.

Generation of VL domain containing CDR3 sequence, *i.e.* CDR1-CDR3 monomer – amino acid sequences of the sdAbs constructs, *i.e.* CDR1-CDR3

monomer were designed by Frederico Aires-da-Silva and Zélia Gouveia based on determined sequences of the sdAbs 1A6 and 2H7. Briefly, the sequence of the CDR1 of sdAbs 1A6 or 2H7 was inserted in the region of the CDR3 of the sdAbs 1A6 or 2H7, generating the represented sdAbs constructs (**Fig. 1**). The gene sequence of these constructs was synthesized by the company NZYtech with a *E.coli* codon usage optimized and cloned into a pUC57 vector. The sequences of each construct were flanked by *NheI* and *XhoI* (New England Biolabs) sites. The DNA sequence of the constructs was digested in *NheI* and *XhoI* and cloned into a bacterial expression vector pET28a. The sdAbs used in this work were characterized in the Chapter 2.

Generation of single chain variable fragments (scFv) with the format VL-VL containing CDR3, *i.e.* CDR1-CDR3 dimer 1A6-2H7 (Fig. 1) – The amino acid sequence of the these sdAbs constructs, *i.e.* CDR1-CDR3 monomer was designed by Frederico Aires-da-Silva and Zélia Gouveia based on determined sequences of the sdAbs 1A6 and 2H7. A linker encoding the 28 amino acid; G T E L E I L S G G G G S G G G G S G G G G S S G R L L was used to connect the two sdAbs CDR1-CDR3 monomer 1A6-2H7, in which the CDR3 sequence of the sdAb 1A6 contains the sequence corresponding to the CDR1 sequence of sdAb 2H7 (**Fig. 1**). The gene sequences of these constructs were synthesized by the company NZYtech with an *E.coli* codon usage optimized and cloned into a pUC57 vector. The cloning into bacterial expression vector pET28a was performed as above. The sdAbs used in this work were characterized in the Chapter 2.

Generation of single chain variable fragments (scFv) with the format VL- VL based on the original sequence of the sdAb 1A6 and 2H7, *i.e* CDR1 Dimers (Fig. 1) – For first sdAb, each sdAbs 1A6 and 2H7 DNA sequence was amplified by specific oligonucleotide primers covering all their sequence. The forward primer was preceded by a *Nhe I* site, while the reverse primer was followed by a linker encoding the 28 amino acid; G T E L E I L S G G G G S G G G G S G G G G S S G R L L and *NotI* and *XhoI* site. The amplified sequence was purified, digested and

cloned into the *NheI*/*XhoI* (New England Biolabs) sites of the bacterial expression vector pET28a. For DNA sequence amplification of the second sdAb, other specific oligonucleotide primers covering each of sdAbs 1A6 and 2H7 DNA sequences were used. These include, a forward primer preceded by a *NotI* site and a reverse primer followed by a stop codon and an *XhoI* site. These sequences were purified, digested and cloned into the *NotI*/*XhoI* sites of the pET28a containing the sequence of the first sdAb. The sdAbs used in this work were characterized in the Chapter 2.

SdAb constructs expression and purification – The sdAbs constructs cloned in a pET28a expression vector were transformed in the strains *E. coli* BL21-CodonPlus(DE3), Tuner and SHuffle T7 (*NEB*) cells and sequences were confirmed (Macrogen DNA Sequencing service), before expression and further purification. For protein expression in small scale, a 3 mL of Super Brown (SB) medium was inoculated with one colony of the transformed bacteria with pET28a -constructs plasmids with the appropriated antibiotic (Kan) grown to exponential phase (λ_{600nm}=0.6-0.9) at 37ºC. Expression was induced by the addition of 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and growth during overnight at 37ºC. Cells were harvested by centrifugation (2800g for 15 min at 4 ºC) and resuspended in 500 µL of 20% BugBuster® Master Mix (Novagen) in PBS supplemented with protease inhibitors (Roche). The protein extract was collect from the supernatant by centrifugation (2800g, 15 min., 4ºC). The expression of the protein was evaluated by ELISA using anti-Human influenza hemagglutinin (HA) as the sdAbs contain a HA tag. For protein expression in large scale, one liter of LB, containing 50 µg/mL carbenicillin was inoculated with 10 mL of overnight culture of SHuffle T7 bacteria cells transformed with pET28a -constructs plasmids and grown to exponential phase (λ_{600nm}=0.6-0.9) at 37ºC. Expression was induced by the addition of 1mM IPTG and growth during 6 h at 37ºC. Cells were harvested by centrifugation (4000g for 15 min at 4 ºC) and resuspended in 50 mL equilibration buffer (50 mM HEPES, 1 M NaCl, 5 mM $CaCl_2$, 10 mM imidazole, pH 7.8) supplemented with protease inhibitors (Roche). Cells were lysed by sonication (20min., on ice) and the inclusion bodies containing the sdAbs were recovered by centrifugation (12096g, 30 min.,

4ºC). Then, the pellet was washed (50mM HEPES, 1M NaCl,10mM imidazole, 5mM $CaCl_2$, 2M urea, 1mM β-mercaptoethanol (β-ME) pH 7.8), sonicated (20 min., on ice) and collected by centrifugation (12096g, 30 min., 4ºC). The inclusion bodies were then re-suspended in a 6 M Urea buffer containing benzonase (>250U, Sigma)(50mM HEPES, 1M NaCl, 10mM Imidazole, 5mM $CaCl_2$, 6M urea, 1mM β-ME, pH 8.0) and incubated overnight (4ºC, under agitation) for protein denaturation. Next day, the denatured sdAbs were purified under denaturing conditions using a His Trap Fast Flow (FF) column (GE Healthcare), and washed twice with 20 and 30 mM of imidazole to remove contaminants (*i.e.* non specific proteins)(50 mM HEPES, 1M NaCl, 6M Urea, 1mM β-ME, 5mM $CaCl_2$, and 20 or 30 mM Imidazole pH 7.8). The sdAb were eluted in highly concentrated imidazole buffer (50 mM HEPES, 1M NaCl, 500 mM Imidazole, 6M urea, 1mM β-ME, 5mM $CaCl_2$, pH 7.8). Re-folding was done using a method for fast removal of the denaturing buffer to non denaturing (50 mM Tris-HCl, 200 mM NaCl, 0,1 mM EDTA, 10% Glycerol, pH 8.5), namely desalting PD10 desalting to promote protein folding. The refolded proteins were then centrifuged to remove protein precipitates (4000g, 4ºC, 10 min.), aliquoted and stored (-80ºC) until used. The purity of the sdAbs was assessed by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with 15% acrylamide composition under denaturating conditions followed by based coomassie stain (Instant Blue, Gentaur, 30 min., RT) or by Western blotting using an Horseradish labeled anti-HA (clone 3F10, Roche).

Protein expression analysis by ELISA – SdAb constructs collected from the supernatant of small scale expression were incubated in a 96 well ELISA plates (Costar 3690) (2h, RT), washed (3x, PBS, 0.1% Tween 20) and blocked in protein free blocking buffer (Pierce; 1h, RT). The sdAbs constructs were detected using a rat anti-HA mAb (3F10; Roche; 0.1µg/mL in protein-free blocking buffer). Plates were washed (5x, PBS, 0.1% Tween 20) and the primary mAb was detected using an alkaline phosphatase labeled rabbit anti-whole rat IgG (Sigma) polyclonal Ab (1 hour, RT) diluted (1/2000) in protein free blocking buffer. Alkaline phosphatase was

revealed with para-Nitrophenylphosphate (pNPP, 1mg/mL; Sigma). To evaluate the total amount of protein, sdAb were coated in 50 mM carbonate/bicarbonate, pH 9.6 (1h; RT or 16; 4ºC) in 96 well ELISA plates (Costar 3690) and the rest of the procedure was performed as described above. Absorbance was measured in a microplate reader at λ_{450nm} (Vitor 3 luminometer; Perkin Elmer).

ELISAs to screen sdAb constructs for heme recognition - 96 well ELISA plates (Costar 3690) were coated with hemin (100µM) in carbonate buffer (0.1M $NaHCO_3$, pH 8.6)(1h, RT or overnight at 4ºC), washed (3x, PBS, 0.1% Tween 20) and blocked in protein free blocking buffer (Pierce; 1h, RT). Plates were washed (4x, PBS, 0.1% Tween 20) and purified sdAbs diluted in PBS were added (1h 30 min., RT). Plates were washed (5x PBS, 0.1% Tween 20) and heme-bound sdAbs were detected using a rat anti-HA mAb (3F10; Roche; 0.1µg/mL in protein-free blocking buffer). Plates were washed (5x PBS, 0.1% Tween 20) and the primary mAb was detected using an alkaline phosphatase labeled rabbit anti-whole rat IgG (Sigma) polyclonal Ab (1 hour, RT) diluted (1/2000) in protein free blocking buffer (Pierce). Alkaline phosphatase was revealed with para-Nitrophenylphosphate (pNPP, 1mg/mL; Sigma). To evaluate the total amount of protein, sdAb were coated in 50 mM carbonate/bicarbonate, pH 9.6 (1h; RT or 16; 4ºC) in 96 well ELISA plates (Costar 3690) and the rest of the procedure was performed as described above. Absorbance was measured in a microplate reader at λ_{450nm} (Vitor 3 luminometer; Perkins Elmer).

Heme competition assays – SdAbs (2.5 µg/mL) constructs were pre-incubated (90 min., RT, mild agitation) with different tetrapyrroles or hemoproteins and ELISA was performed as described above. Concentrations of tetrapyrroles or hemoproteins used for pre-incubation are expressed as heme molar equivalents.

Biacore – The affinities of sdAbs constructs towards heme were obtained using surface plasmon resonance (SPR)(BIAcore 2000, BIAcore Inc.). Briefly, sdAbs were captured on a CM5 chip using amine coupling at ~800 resonance units (RU).

Heme at 0-4000 nM was injected for 4 min and sdAb-bound heme was allowed to dissociate (10 min.) before matrix regeneration (10 mM Glycine, pH 2.5). The signal was subtracted from that of an immobilized cell to generate sensorgrams of the amount of bound heme as a function of time. The running buffer, HBS-P (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20; BIAcore) was used for all sample dilutions. BIAcore kinetic evaluation software (version 3.1) was used to determine dissociation constant (KD) from the association and dissociation rates using a one-to-one binding model. An irrelevant sdAb was used as negative control.

6. ACKNOWLEDGEMENTS

The entire inflammation group at Instituto Gulbenkian de Ciência for critical discussions and comments. This work was supported by Fundação para a Ciência e Tecnologia (RECI-IMI-IMU-0038-2012; PTDC/SAU-TOX/116627/2010; HMSP-ICT/0018/2011 to MPS, SFRH/BD/44828/2008 to ZG, PTDC/SAU-FAR/119173/2010 to JG), from ERC-2011-AdG 294709-DAMAGECONTROL to MPS.

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ITQB-UNL | Av. da República, 2780-157 Oeiras, Portugal
Tel (+351) 214 469 100 | Fax (+351) 214 411 277

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