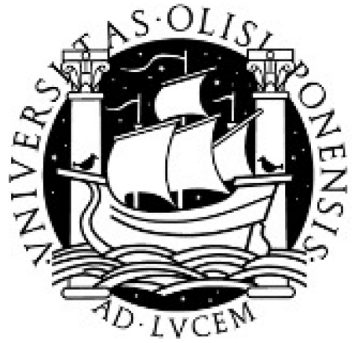
Universidade de Lisboa

Faculdade de Medicina



Role of the Protective gene Heme Oxygenase-1 in the control of T cell mediated responses

by

Ângelo António Ferreira Chaves do Rosário Chora

Tese submetida para obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Ciências Biopatológicas

2008

Ângelo António Ferreira Chaves do Rosário Chora

Role of the Protective gene Heme Oxygenase-1 in the control of T cell mediated responses

Tese submetida para obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Ciências Biopatológicas

Orientadores:

Doutor Miguel Soares

Instituto Gulbenkian de Ciência

Professora Doutora Leonor Parreira

Faculdade de Medicina da Universidade de Lisboa

2008

As opiniões expressas são da exclusiva responsabilidade do autor

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 11 de Dezembro de 2007

Aos meus dois pilares:

Patrícia e pai.

Em memória da minha mãe.

L'enchantement de la science consiste en ce que, partout et toujours nous pouvons donner la justification de nos principes et la preuve de nos découvertes.

Louis Pasteur, 1882

Table of contents

Acknowledgments

Sir Winston Churchill (1874 - 1965) is quoted as having said: "*Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb."* At this point, it is the time to acknowledge those who, by climbing with me, helped me along the way or just by standing on the side of my *scientific* path contributed in a crucial manner to the successful completion of my PhD. All that I've received and learned from the people listed below will always be present throughout the entire length of my life´s journey.

My PhD. supervisor Miguel Soares. There are many things for which I am indebted to you not only as a researcher but also as a person. Only with your scientific input, trust, never ending enthusiasm and support was it possible for me to develop a piece of work, now summarized in this thesis, of which I am very proud. I am particularly grateful for the many interesting and rewarding discussions over the years where, even if not always agreeing or maybe exactly because of that, you pushed me a step forward and made me take a stand.

All former and current laboratory colleagues. I would have to write as many pages as in this Thesis to acknowledge all that each and every one of you was kind enough to offer me. Throughout these 5 years you were my family outside home. As a group, I am thankful for your team spirit and unselfishness and truly appreciate the advices and inspiring criticism I received from all of you. I also hold particular debts. To Sofia Rebelo for, while being the *lab mummy,* indulging me in my chaos always with a charming smile. To Andreia Cunha for being an excellent person not only to share the bench with but also, and perhaps more importantly, to share so many hours of mutual company. To Nadja Pejanovic for all the conversations of *no consequence and yet so important.* To Sílvia Cardoso for all the time she waited for me to arrive in the morning, sometimes at her stomach expenses, so that I could enjoy my breakfast in her company. To Isabel Gregoire for her willingness to help in every occasion and for being a dedicated listener. To Tatiana Vassilievskaya who helped me in the beginning of this work and never failed to encourage me in difficult times. To Gabriela Silva, Rasmus

Larsen, Ana Ferreira, Mark Seldon, Lazlo Tokaji, Moisés Marinho, Rafaella Gozzellini, Eliane Cortez and Nuno Palha for putting up with me and making lab life easier with *comfort* coffee breaks, lunches and much more.

All the people that I was fortunate enough to collaborate with or that helped me with specific tasks. To Paulo Fontoura for being always available and ready to help with critical input and teaching me the techniques necessary to pursue this project. To Lawrence Steinman, Peggy Ho, Lowen Lee, Raymond Sobel, Santiago Zelenay, Jocelyne Demongeot, Ana Pamplona and Maria Mota for very fruitful collaborations. To Teresa Pais, Santiago Zelenay, Jocelyne Demongeot, Iris Caramalho and Margarida Vigário for sharing not only technical resources but also their knowledge with me as well as for being the pleasantest neighbors one could ask for. To Dolores Bonaparte who was tireless in providing all the animals and the animal facility services necessary for the completion of this work. To Thiago Carvalho, Santiago Zelenay, Paulo Fontoura and Teresa Pais for reading this manuscript and their insightful input. To my Thesis Committee, Jocelyne Demongeot and Moisés Mallo for all the support.

Fundação para a Ciência e Tecnologia for funding me throughout my scientific carrier and to Instituto Gulbenkian de Ciência for providing excellent working conditions and financial support during the completion of this thesis.

All my friends, thankfully too many to mention their names, either close or far, present every day or together just for a coffee during weekends, from whom I always received the best I could expect. I could not write these pages without expressing my deepest thankfulness for all the generosity, companionship and help all these years and my bewilderment for being worthy of your friendship.

My family, without whom so many things would not have been possible. To Dad, for all the efforts and sacrifices involved in bringing me up and turning me the man I am now. *My faults are mine, not yours*. To my niece, sister and brother-in-law who never failed to support me in all my dreams and pursuits. To Célia, Ami, Avó Boneca, Avó Teresa, Tia Guida, Tia Kiki and all others for continuously

rejoicing with my accomplishments, being always interested in listening to *immunology tutorials* and specially Ami for making my life as easy as possible so that I could devote myself to science.

My wife, Patrícia. There are details easy enough to acknowledge here: the weekends you spent alone while I worked, the re-heated dinners you served because I was late, your rides to work because I don't drive, the hours you spent listening to *lab talk*, and the list could go on and on… And yet, I struggle to lay in these pages the most important things for which I'll be eternally grateful to you. That is because very few people, and unfortunately I am not one of them, can put into words what one takes from one's loved one. My strength, my courage, the awareness of what I am and what I can accomplish, my very best all stem from you and your love. There are no words to express this kind of gratitude. I love you.

Figures and tables

Chapter 1

Chapter 2

Abbreviations

AICD activation-induced cell death
ALAS δ-aminolevulinic acid synthase
APC antigen presenting cell
APL altered peptide ligands
ASC apoptosis-associated speck-like protein containing a CARD
ATP adenosine tri-phosphate
BBB blood brain barrier
BCR B cell receptor
BR bilirubin
BV biliverdin
BVR biliverdin reductase
CCR5 chemokine (C-C motif) receptor 5
CFA complete Freund's adjuvant
cGMP cyclic guanosine monophosphate
CIITA class II transactivator
CM cerebral malaria
CNS central nervous system
CO carbon monoxide
Co cobalt
CO_2 carbon dioxide
COHb carboxyHb
CoPPIX cobalt protoporphyrin IX
COX cycloxygenase
CREB cyclic-AMP-responsive-element-binding protein
CSF cerebrospinal fluid
CSFE 5,6-carboxy-succinimidyl-fluorescein-ester
CTL cytotoxic T lymphocyte
CTLA-4 cytotoxic T-lymphocyte-associated antigen 4
CXCR3 chemokine (C-X-C motif) receptor 3
DAMP danger-associated molecular pattern
DCs dendritic cells
EAE experimental autoimmune encephalomyelitis
EBV Epstein-Barr virus
EC endothelial cell
ECM experimental cerebral malaria
ELISA enzyme-linked immunosorbent assay
eNOS endothelium nitric oxide synthase
ERK extracellular signal-regulated kinase
FACS fluorescent-activated cell sorting
FePPIX iron protoporphyrin IX
Foxp3 forkhead box p 3
GFP green fluorescent protein
GIPL glycoinositolphospholipids
GITR glucocorticoid-induced tumor necrosis factor receptor
GM-CSF granulocyte-macrophage colony-stimulating factor
GPI glycosylphosphatidylinositol
GSH glutathione
HIF-1α hypoxia-inducible factor–1alpha
HLA human leukocyte antigen
Hmox heme oxygenase gene
HO heme oxygenase
Hp haptoglobin
HPRT hypoxanthine-guanine phosphoribosyltransferase
HRP horseradish peroxidase
HSP heat shock protein
Hx hemopexin
ICAM intercellular adhesion molecule
IDO idoleamine 2,3-dioxygenase
IFN interferon
Ig immunoglobulin
IL interleukin
iNOS inducible nitric oxide synthase
IP-10 interferon-gamma-inducible protein
IRF IFN-regulatory factor
JAK Janus kinase
JNK c-Jun N terminal kinase
KA kynurenic acid
LDH lactate dehydrogenase
LDL low-density lipoprotein
LFA-1 lymphocyte function-associated antigen
LN lymph nodes
LOX lipoxygenase
LPS lipopolysaccharide
LRR leucine rich repeat
MAC membrane attack complex
MAG myelin-associated glycoprotein
MAL MyD88-adapter-like
MAPK mitogen-activated protein kinase
MBP myelin basic protein
MDA5 melanoma differentiation-associated gene 5
MHC major histocompatibility complex
MIP macrophage inflammatory protein
MMP matrix metalloproteinases
Mø monocyte/macrophage
MOG myelin oligodendrocyte glicoprotein

MS multiple sclerosis
MyD88 myeloid differentiation primary response gene 88
NADPH nicotinamide dinucleotide phosphate
NALP NACHT-LRR-PYD-containing protein
NFY nuclear transcription factor Y
NF-κB nuclear factor-kappa B
NLR NACHT–leucine-rich repeat
NO nitric oxide
NOD nucleotide binding oligomerization domain
Nrf2 NF-E2-related factor 2
O_2 oxygen
ODC oligodendrocyte
OVA ovalbumine
PAF platelet-activating factor
PAR protease-activated receptor
PBLs peripheral blood lymphocyte
PBS phosphate buffered saline
PCR polymerase chain reaction
PD-1 programmed cell death 1
PG prostaglandin
PKC protein kinase C
PLP proteolipid protein
PMN polymorphonuclear cell
PPARγ peroxisome proliferator-activated receptor gamma
PRR pattern recognition receptor
PSGL1 P-selectin glycoprotein ligand 1
QA quinolinic acid
RAG recombination-activating gene
RANTES regulated upon activation, normally T-expressed, and presumably secreted
RBC red blood cell
RFX regulatory factor X
RIG-I retinoic-acid-inducible protein I
RNA ribonucleic acid
RNS reactive nitrogen species
ROS reactive oxygen species
SAM severe acute malaria
SCF stem cell factor
SCID severe combined immunodeficiency
sGC soluble guanylate cyclase
SMCs smooth muscle cells
Sn tin
SNP single nucleotide polymorphism
SnPPIX tin protoporphyrin IX
SOD superoxide dismutase
SPF specific pathogen free
STAT-1 signal transducer and activator of transcription
T-bet T-box expressed in T cells
TCR T cell receptor
TF tissue factor
TGF-β transforming growth factor beta
T_H T helper
TIRAP TIR-associated protein
TLR toll-like receptor
TMEV Theiler's murine encephalomyelitis virus
TNF tumour necrosis factor
TNF-R1 tumour necrosis factor receptor 1
TRAM TRIF-related adaptor molecule
T_{reg} naturally occurring regulatory $CD4^+$ T cell
TRIF TIR-domain-containing adaptor protein-inducing IFN-β
UDPGT uridine diphosphate glucuronyltransferase
VCAM vascular cell adhesium molecule
VLA-4 very late antigen 4
VSMC vascular smooth muscle cell
Zn zinc
ZnPPIX zinc protoporphyrin

Preface

This Thesis describes the data obtained during the research work performed from October 2002 to July 2007 at the *Instituto Gulbekian de Ciência* under the scientific supervision of Dr. Miguel Soares, PhD.

The Thesis is organized in 5 chapters and 1 annex, which are preceded by a abstract written both in Portuguese and English. An introductory review on the subject is provided in Chapter 1. In Chapters 2, 3, and 4 the original observations obtained during the research period are presented and discussed. Chapter 5 consists in an extended discussion aiming at integrating the results presented in the previous Chapters.

Sumário

As reacções inflamatórias, geralmente desencadeadas por infeccões e/ou lesões a nível dos tecidos, desempenham um papel fundamental na iniciação de respostas imunes adaptativas e conduzem, em última análise, à eliminação do evento instigador. São vários os mecanismos intrínsecos a esta resposta complexa que asseguram, após a remoção do estímulo nocivo, a correcta reparação quer a nível estrutural quer funcional do tecido afectado, ou seja, o regresso à homeostase. A importância destes mecanismos pode ser comprovada pelo facto de a não resolução das reacções inflamatórias ser uma etapa crítica para o estabelecimento e/ou progressão de um número crescente de patologias. Um dos mecanismos envolvidos na resolução da inflamação consiste na expressão do enzima Heme Oxygenase-1 (HO-1). Em condições inflamatórias, a HO-1 torna-se o enzima limitante no catabolismo dos grupos hémicos livres dando origem a quantidades equimolares de monoxido de carbono (CO), ferro (Fe) e biliverdina (BV). Estes produtos reduzem a reacção inflamatória e evitam o desenvolvimento de doenças inflamatórias.

Esta Tese teve como objectivo examinar o papel da HO-1 na regulação do estabelecimento e progressão de condições neuroinflamatórias mediadas por linfócitos T. O trabalho agora apresentado sugere que a HO-1 dita o resultado patológico associado com processos neuroinflamatórios em ratinho, tais como a encefalomielite autoimune experimental (EAE), um modelo de esclerose múltipla (EM), ou a malária cerebral experimental (MCE) resultante da infecção com *Plasmodium spp.*

A indução de EAE em ratinhos com disrupção funcional do gene da HO-1 (*Hmox-1*$^{-/-}$) caracterizou-se por um aumento da desmielinização do sistema nervoso central (SNC), assim como da paralisia e mortalidade associadas, por comparação com ratinhos com o gene intacto (*Hmox-1*$^{+/+}$). A indução farmacológica da expressão da HO-1, após os sinais clínicos associados com a EAE serem evidentes, resultou na melhoria da progressão clínica da doença. Demonstrou-se que este efeito está associado a uma inibição da acumulação, proliferação e função efectora de linfócitos T *helper* e de linfócitos T CD8^{+} no SNC. Neste contexto, o efeito protector da HO-1 não foi mediado pela modulação da actividade supressora de linfócitos T regulatórios, responsáveis pela tolerância periférica a auto-antigénios e pela homeostase imunológica. Acresce que, em

condições homeostáticas, o desenvolvimento, manutenção e função dos linfócitos T regulatórios são independentes da HO-1 de acordo com o estudo efectuado em ratinhos *Hmox-1*$^{-/-}$. Demonstrou-se ainda que o mecanismo subjacente ao efeito protector da HO-1 depende da sua capacidade de inibir a expressão do complexo de histocompatibilidade classe II nas células apresentadoras de antigénio, incluindo células dendríticas, microglia e células da linhagem macrofágica. Do mesmo modo, a inactivação de *Hmox-1* ou a inibição farmacológica da actividade da HO-1 resultaram num aumento da incidência de MCE em estirpes de ratinho resistentes, ao passo que a indução farmacológica da enzima reduziu significativamente a incidência de MCE em estirpes susceptíveis. A inducção farmacológica da expressão da HO-1 diminuiu o sequestro de linfócitos T CD8^{+} na microvasculatura do SNC, um evento crítico para o desenvolvimento de lesões neurológicas associadas com MCE.

Em ambas as patologias (EAE e MCE) a administração de CO exógeno simulou estes efeitos protectores, o que sugere que este gás é o principal mediador da actividade protectora da HO-1. Por último, são apresentadas evidências de um novo mecanismo pelo qual o CO impede o desenvolvimento de uma destas patologias (MCE) através da sua capacidade para se ligar à hemoglobina, evitar a sua oxidação e consequente libertação de moleculas efectoras centrais na patogénese de MCE, i.e. os grupos hémicos.

Globalmente, as observações apresentadas nesta Tese sugerem que durante o estabelecimento e/ou progressão de processos neuroinflamatorios, o enzima HO-1 e/ou o CO limitam as respostas imunes prejudiciais associadas à neuroinflamação, possivelmente através da modulação da actividade das células apresentadoras de antigénio. Estas observações fundamentam o uso da indução farmacológica da HO-1 ou a administração de CO como potenciais estratégias para o tratamento de doenças neuroinflamatórias.

Summary

Inflammatory reactions, elicited in most cases upon infection and/or injury, are critical for the initiation of adaptive immunity and ultimately lead to the removal of the inciting stimuli. Intrinsic to this complex response, there are several mechanisms that operate to ensure that, once the inciting stimulus is dealt with, structural and functional repair of the injured site is attained, i.e. return to homeostasis. However, failure to resolve inflammatory reactions is thought to contribute in a critical manner to the establishment and/or progression of a growing list of pathologic conditions. One of the mechanisms involved in the resolution of inflammation relies in the expression of Heme Oxygenase (HO)-1. Under inflammatory conditions, HO-1 becomes the rate-limiting enzyme in the catabolism of heme, yielding equimolar amounts of carbon monoxide (CO), free iron (Fe) and biliverdin (BV). These heme degradation products dampen inflammation and prevent the development of inflammatory diseases.

The focus of this Thesis was to address whether HO-1 regulates the establishment and progression of T cell-mediated neuroinflammatory conditions. The body of work presented herewith suggests that HO-1 can dictate the pathologic outcome of neuroinflammation in mice, as it occurs either during autoimmunity-driven experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), or during experimental cerebral malaria (ECM) triggered upon infection with *Plasmodium spp*. Induction of EAE in HO-1-deficient (*Hmox-1*$^{-/-}$) mice led to enhanced central nervous system (CNS) demyelination, paralysis and mortality, as compared to wild-type (*Hmox-1*$^{+/+}$) mice. Pharmacological induction of HO-1 expression after EAE onset improved the clinical course of the disease, an effect associated with inhibition of T helper (T_H) and $CD8^+$ T cell accumulation, proliferation and effector function within the CNS. HO-1 did not act via modulation of the suppressor activity of naturally occurring regulatory T cells (T_{reg}), known to ensure peripheral tolerance to self-antigens and immune homeostasis. Furthermore, under homeostatic conditions T_{reg} development, maintenance and function were found to be independent of HO-1, as assessed in *Hmox-1*$^{-/-}$ mice. Instead, the mechanism underlying the protective effect of HO-1 is shown to rely on its ability to inhibit major histocompatibility complex (MHC) class II expression by antigen presenting cells, including dendritic cells, microglia and macrophages.

Likewise, *Hmox-1* deletion or pharmacological inhibition of its activity resulted in increased ECM incidence in otherwise resistant mouse strains whereas pharmacological induction of HO-1 greatly reduced ECM incidence in susceptible mouse strains. The protection afforded by pharmacological induction of HO-1 expression was associated with decreased $CD8^+$ T cell sequestration in the CNS, a critical event in the development of neurological damage associated with ECM. In both pathologies, i.e. EAE and ECM, exogenous CO mimicked these protective effects, suggesting that CO is the main contributor to the protective action of HO-1. Finally, we present evidence of a novel mechanism by which CO counters the development of one of these pathologies, ECM, based on its ability to bind hemoglobin, prevent its oxidation and subsequently the generation of free heme, a central effector molecule in the pathogenesis of ECM.

Overall, the findings presented in this Thesis suggest that, during the establishment and/or progression of neuroinflammation, HO-1 and/or CO limit the deleterious effects associated with neuroinflammatory responses, possibly by modulating antigen presenting cells activity, in a manner that prevents the pathologic outcome of these conditions. Further, these results support the notion that pharmacological modulation of HO-1 or CO administration might be potential therapeutic strategies to counter neuroinflammatory diseases.