

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

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Faculdade de Medicina da Universidade de Lisboa

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As opiniões expressas são da exclusiva responsabilidade do autor

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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

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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.

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Abbreviations

AICD	activation-induced cell death	GFP	green fluorescent protein
ALAS	δ -aminolevulinic acid synthase	GPI	glycoinositolphospholipids
APC	antigen presenting cell	GITR	glucocorticoid-induced tumor necrosis factor receptor
APL	altered peptide ligands	GM-CSF	granulocyte-macrophage colony-stimulating factor
ASC	apoptosis-associated speck-like protein containing a CARD	GPI	glycosylphosphatidylinositol
ATP	adenosine tri-phosphate	GSH	glutathione
BBB	blood brain barrier	HIF-1α	hypoxia-inducible factor-1 alpha
BCR	B cell receptor	HLA	human leukocyte antigen
BR	bilirubin	Hmox	heme oxygenase gene
BV	biliverdin	HO	heme oxygenase
BVR	biliverdin reductase	Hp	haptoglobin
CCR5	chemokine (C-C motif) receptor 5	HPRT	hypoxanthine-guanine phosphoribosyltransferase
CFA	complete Freund's adjuvant	HRP	horseradish peroxidase
cGMP	cyclic guanosine monophosphate	HSP	heat shock protein
CIITA	class II transactivator	Hx	hemopexin
CM	cerebral malaria	ICAM	intercellular adhesion molecule
CNS	central nervous system	IDO	idoleamine 2,3-dioxygenase
CO	carbon monoxide	IFN	interferon
Co	cobalt	Ig	immunoglobulin
CO₂	carbon dioxide	IL	interleukin
COHb	carboxyHb	iNOS	inducible nitric oxide synthase
CoPPIX	cobalt protoporphyrin IX	IP-10	interferon-gamma-inducible protein
COX	cyclooxygenase	IRF	IFN-regulatory factor
CREB	cyclic-AMP-responsive-element-binding protein	JAK	Janus kinase
CSF	cerebrospinal fluid	JNK	c-Jun N terminal kinase
CSFE	5,6-carboxy-succinimidyl-fluorescein-ester	KA	kynurenic acid
CTL	cytotoxic T lymphocyte	LDH	lactate dehydrogenase
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4	LDL	low-density lipoprotein
CXCR3	chemokine (C-X-C motif) receptor 3	LFA-1	lymphocyte function-associated antigen
DAMP	danger-associated molecular pattern	LN	lymph nodes
DCs	dendritic cells	LOX	lipoxygenase
EAE	experimental autoimmune encephalomyelitis	LPS	lipopolysaccharide
EBV	Epstein-Barr virus	LRR	leucine rich repeat
EC	endothelial cell	MAC	membrane attack complex
ECM	experimental cerebral malaria	MAG	myelin-associated glycoprotein
ELISA	enzyme-linked immunosorbent assay	MAL	MyD88-adaptor-like
eNOS	endothelium nitric oxide synthase	MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase	MBP	myelin basic protein
FACS	fluorescent-activated cell sorting	MDA5	melanoma differentiation-associated gene 5
FePPIX	iron protoporphyrin IX	MHC	major histocompatibility complex
Foxp3	forkhead box p 3	MIP	macrophage inflammatory protein
		MMP	matrix metalloproteinases
		Mϕ	monocyte/macrophage
		MOG	myelin oligodendrocyte glycoprotein

MS	multiple sclerosis	SPF	specific pathogen free
MyD88	myeloid differentiation primary response gene 88	STAT-1	signal transducer and activator of transcription
NADPH	nicotinamide dinucleotide phosphate	T-bet	T-box expressed in T cells
NALP	NACHT-LRR-PYD-containing protein	TCR	T cell receptor
NFY	nuclear transcription factor Y	TF	tissue factor
NF-κB	nuclear factor-kappa B	TGF-β	transforming growth factor beta
NLR	NACHT-leucine-rich repeat	T_H	T helper
NO	nitric oxide	TIRAP	TIR-associated protein
NOD	nucleotide binding oligomerization domain	TLR	toll-like receptor
Nrf2	NF-E2-related factor 2	TMEV	Theiler's murine encephalomyelitis virus
O₂	oxygen	TNF	tumour necrosis factor
ODC	oligodendrocyte	TNF-R1	tumour necrosis factor receptor 1
OVA	ovalbumine	TRAM	TRIF-related adaptor molecule
PAF	platelet-activating factor	T_{reg}	naturally occurring regulatory CD4 ⁺ T cell
PAR	protease-activated receptor	TRIF	TIR-domain-containing adaptor protein-inducing IFN- β
PBLs	peripheral blood lymphocyte	UDPGT	uridine diphosphate glucuronyltransferase
PBS	phosphate buffered saline	VCAM	vascular cell adhesion molecule
PCR	polymerase chain reaction	VLA-4	very late antigen 4
PD-1	programmed cell death 1	VSMC	vascular smooth muscle cell
PG	prostaglandin	Zn	zinc
PKC	protein kinase C	ZnPPiX	zinc protoporphyrin
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		

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 infecções 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 monóxido 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 rato, 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-antígenos 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 macrófágica. 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 indução 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 moléculas 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 neuroinflamatórios, 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.