Chapter 1: General Introduction.

1. Innate and adaptive immune responses

Aulus Cornelius Celsus defined inflammation in AD40 as "*calor, rubor, tumor et dolor*". Later, "*loss of function*" was added as the fifth cardinal sign. In a broad sense, this definition is still accepted today if one associates *calor* and *rubor* with increased blood flow in microvasculature, *tumor* with increased microvascular permeability to plasma proteins and leukocyte extravasation and *dolor* with the release of neuromediators that induce pain. The modern definition of inflammation encompasses a series of sequential responses of vascularized tissue to damage caused either by injurious chemicals, physical insults or microbial pathogens. These responses consist of a regulated network of events including alterations in hemodynamics and vascular permeability, release of molecular mediators and the involvement of cellular components (e.g. innate immune cells) leading to the elimination of the inciting stimulus and to structural and functional repair of the injured site, i.e. return to homeostasis. Overall, inflammation should be perceived as a salutary response, critical for the initiation of immune responses that will ultimately lead to microbial clearance. This notion is strongly supported by the observation that individuals with genetic deficiencies in major components of the inflammatory process can suffer from recurrent infections, often with deadly consequences[20].

In the majority of cases, a protective response against infection and/or injury involves the innate and the adaptive arms of the immune system. Innate responses, characterized by their immediate and relatively non-specific character, involve mechanisms aiming at dealing with the injurious insult in a generic manner. Cellular components of the innate immune system express germline-encoded receptors, named pattern recognition receptors (PRRs). These recognize molecules associated with the occurrence of infection and/or tissue injury, collectively termed danger associated molecular patterns (DAMPs). Following DAMP recognition, PRRs can trigger a series of signal transduction pathways leading to profound modifications of the cellular phenotype, aiming at adapting cellular function to the challenges imposed by infection and/or tissue damage.

In addition from providing the first line of defense against infectious microorganisms, innate immunity is also instrumental in the activation of the adaptive immune system, a notion that underscores the interdependency between the two systems. The generation of an effective immune response against a given pathogen requires two major cellular subsets, i.e. antigen presenting cells

(APCs) provided by the innate system and lymphocytes, i.e. B and T cells. While PRRs can directly activate B cells, T cell activation is dependent on PRR engagement in APCs. This is underscored by the capacity of PRR ligands to act as immunologic adjuvants, that is to promote immune responses despite their lack of antigenic specificity. These events culminate in the productive activation of the adaptive counterpart of the immune system, eliciting a specific and robust reaction as inflammation develops into an immune response. In turn, the adaptive arm of the immune system influences the action of the innate system, in a way as to deal with the initiating noxious stimulus. As such, both the innate and adaptive immune systems act together, via a coordinated interplay, to eradicate the cause of injury.

1.1. Kinetics of inflammatory reactions

The progression of inflammatory responses involves a well-orchestrated sequence of events and can be divided in *initiation*, *effector* and *resolution* phases. Each phase is tightly regulated. In the early stages of inflammation, regulation relies on the integration of signals that preclude defaulting to the steady state before the source of injury is removed. In later phases, the antimicrobial and tissue-damaging nature of the reaction is converted into one that promotes repair and return to homeostasis.

1.1.1. Initiation phase

Although soluble molecules, e.g. complement, pentraxins, etc, can contribute to the initiation of inflammatory responses, bone marrow-derived leukocytes of the innate immune system are critical to this process. Given their widespread distribution throughout the body and constant transiting between the blood stream and tissues, leukocytes of the innate immune system, including neutrophils, monocytes/macrophages (Mø) and mast cells, constitute the first line of defence against microbial infections and thus play a critical role in the so called immune surveillance.

Leukocytes of the innate immune system can recognize a vast range of highly conserved microbial and presumably endogenously driven DAMPs. Recognition occurs via germ-line encoded PRRs (Figure 1.1). These exhibit distinct ligand specificities and expression patterns and can activate specific signal transduction pathways, leading to the coordinated expression of a plethora

of genes involved in the initiation of inflammatory responses. The best characterized family of PRRs are Toll-like receptors (TLRs), a group of evolutionarily conserved receptors expressed in plants, insects and vertebrates, encompassing at least 13 paralogues in mammals, 10 of which are expressed in humans[21]. TLR1, 2, 4, 5 and 6 are positioned at the cell surface, recognizing extracellular DAMPs. TLR3, 7, 8 and 9 associate exclusively with the luminal side of endosomal vesicles and are involved in DAMP sensing following cellular internalisation. Other surveillance

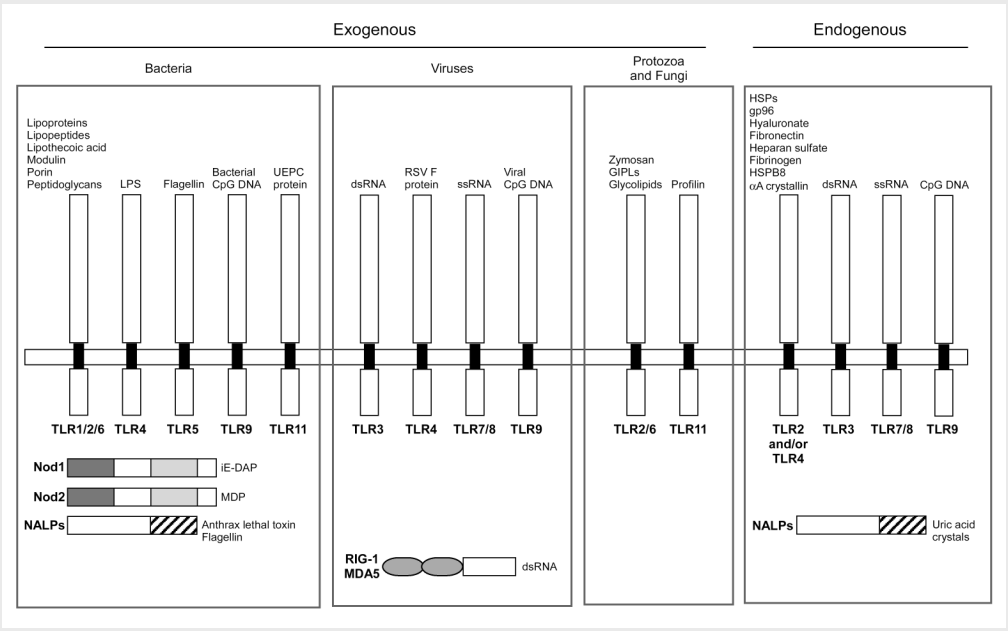

Figure 1.1 – **PRR receptors and their ligands**. Numerous PRR ligands, either from bacterial, viral, protozoan, fungi or endogenous origin have been identified. Detection of Gram-positive bacteria involves TLR2 homodimers or functional associations of this receptor with TLR1 and TLR6. TLR2 homodimers can recognize peptidoglycans, modulin and porin while heterodimeric TLR2/1 and TLR2/6 bind lipopeptides and lipoteichoic acid, respectively. TLR4 recognizes LPS, a component of Gram-negative bacteria cell walls, TLR5 binds to flagellin and TLR11 detects a protein from uropathogenic bacteria. DNA hypomethylation in the context of a specific base sequence, the so-called CpG motif, is recognized by TLR9. Viral recognition is provided by TLR3, TLR4, TLR7/8 and TLR9 through binding to double-stranded (ds)RNA, respiratory syncytial virus fusion protein (RSVF P), single-stranded (ss)RNA and the CpG DNA motif, respectively. Protozoan glycolipids (GIPLs) and fungi-derived zymosan are sensed by heterodimeric TLR2/6 while the protozoan profiling-like protein is recognized by TLR11. In what concerns endogenous ligands, TLR2 homodimers can recognize HSP60 while TLR4 recognizes endogenous HSP60, 70 and 90, components of the extracellular matrix including fibronectin, hyaluronate, and heparan sulfate, as well as fibrinogen. TLR3, 7/8 and 9 sense endogenous nucleic acids[8, 9]. Another class of PRRs, consisting in the NLR family of proteins, currently with 22 identified members, are involved in detection of cytosolic DAMPs. Two of the best characterized members of the NLRs are Nod1 and Nod2. Nod1 senses γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) found predominantly in Gram-negative bacteria[14] while Gram-positive and Gram-negative muramyl dipeptide (MDP) is the specific ligand responsible for Nod2 activation[16]. Yet another class of the NLR family of cytoplasmatic receptors, NALPs, are activate upon recognition of ligands that include anthrax lethal toxin, host-derived ureic acid crystals and bacterial flagellin[17]. In addition, the RNA helicases RIG-I and MDA5 recognize viral dsRNA within the cytoplasmatic compartment[18, 19].

mechanisms have evolved to detect DAMPs in subcellular locations not readily accessible to TLRs. One of such mechanisms relies on the detection of DAMPs within the cytosol by the RNA helicases retinoic-acid-inducible protein I (RIG-I)[18,] and melanoma differentiation-associated gene 5 (MDA5)[19]. Another family of cytoplasmatic PRRs are the NACHT–leucine-rich repeat (NLR) family, whose members encompass NACHT-, leucine-rich repeats (LRR)-, and pyrin domain-containing proteins (NALPs)[22] and the nucleotide binding oligomerization domain–LRR (NOD-LRR) protein family[23].

The molecular mechanisms underlying PRR activity depend on the formation of molecular complexes that initiate signal transduction pathways in a way as to activate the expression of genes required for an effective inflammatory response. In the case of TLRs, ligand recognition leads to the formation of homo or heterodimers with other TLR members and/or with specific accessory proteins, e.g. CD14. The conformational modifications associated with DAMP/TLR interactions promote the recruitment of intracellular signalling adapter molecules. The combinatorial effect owing to the use of four possible adapter molecules, namely myeloid differentiation primary response gene 88 (MyD88), TRIF-related adaptor molecule (TRAM), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) and TIR-associated protein (TIRAP)/MyD88-adapter-like (MAL)[24], can explain, at least partially, the activation of different signal transduction pathways leading to specific biological responses. Signal transduction by TLRs can culminate in the activation of transcription factors of the nuclear factor-kappa B (NF-κB)[8] family that promote the transcription/expression of several pro-inflammatory genes encoding adhesion molecules, cytokines or chemokines. Alternatively, other transcription factors activated via TLR ligation include interferon (IFN)-regulatory factor (IRF)3 and IRF7, responsible for the induction of INF-α, INF-β and IFN-inducible genes[8]. In addition, TLR signaling activates the mitogen-activated protein kinases (MAPKs) extracellular signal–regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)[24] known to participate in the transcriptional activation of pro-inflammatory genes.

In what concerns cytosolic PRRs, oligomerization of Nod1 and Nod2, the best characterized members of the NOD family, also results in activation of NF-κB[25, 26]. PRRs of the NALP family do not seem to induce NF-κB-dependent transcription. Instead, these receptors induce the formation of a molecular complex, the inflammasome, leading to the activation of the pro-inflammatory

caspase-1 and –5 leading to the cleavage/maturation of pro-interleukin (IL)-1β and pro-IL-18 into their bioactive forms[27], a central event in the initiation of inflammatory responses.

Once activated via PRR signaling, leukocytes of the innate immune system release a plethora of soluble mediators that coordinate the onset of an inflammatory response. Some of these can be released immediately upon PRR signalling such as histamine, serotonin, lysosomal hydrolases. Alternatively, others are synthesized *de novo* from metabolism of phospholipids and arachidonic acid, including prostaglandin (PG)E_2, leukotrienes, thromboxanes, and platelet-activating factor (PAF).

Other soluble molecules produced upon PRR ligation include vasodilatory factors that promote widening of the microvasculature and increased blood flow thus facilitating the access of circulating leukocytes to the inflamed tissues and their interaction with the endothelium. This latter set includes the gas nitric oxide (NO), generated primarily in Mø by the inducible form of nitric oxide synthase (iNOS), whose transcription is up-regulated upon NF-kB activation. In addition, NO is also generated via endothelium NO synthase (eNOS) constitutively expressed in endothelial cells (EC). NO promotes vasodilation via a mechanism that targets vascular smooth muscle cells (VSMC) and activates guanylate cyclase, an enzyme responsible for the generation of cyclic guanosine monophosphate (cGMP), a potent vasodilator.

Simultaneously to vasodilation, there is activation of the coagulation cascade with consequent fibrin formation in a mechanism intended to confine the noxious stimuli and the ensuing inflammatory reaction to a limited area. The main initiator of inflammation-induced coagulation is tissue factor (TF), a transmembrane protein whose expression is induced in innate immune cells and ECs by molecular mediators, namely IL-6, in a way as to promote coagulation[28]. In addition, TF is normally expressed on cells at biological boundaries such as skin, organ surfaces, vascular adventitia and epithelial surfaces but not on cells within the vasculature[29]. Inflammation-mediated disruption of vascular integrity exposes TF-expressing cells to circulating blood and promotes the formation of a molecular complex that culminates in the generation of thrombin, a serine protease responsible for fibrin polymerization from fibrinogen. This, coupled with platelet activation, results in the formation of a fibrin-platelet thrombotic clot. Recent evidence suggests that both systems

interact in a way that inflammation not only triggers the coagulation pathway, but coagulation also substantially modulates inflammation[30].

Another key event in the initiation of an inflammatory response is the recruitment and extravasation of leukocytes into the site of infection/injury, a process regulated by both chemokines and adhesion molecules. Chemokines and chemokine receptors have a crucial role in directing leukocyte locomotion. During inflammation, chemokines are produced and attract leukocytes expressing their cognate receptors to the injured site by establishing extracellular gradients. The activation of chemokine receptors in leukocytes initiates signalling pathways that ultimately lead to cell polarization and directed motility. Concomitantly, the release of pro-inflammatory cytokines by activated Mø and mast cells results in the conversion of EC that line the blood vessels from an anti-adhesive and anti-thrombotic state to a pro-adhesive and pro-thrombotic state that facilitates the movement of circulating leukocytes to the site of inflammation. Transmigration across ECs is preceded by interaction between leukocytes and ECs in a process regulated by the expression of adhesion molecules. This heterogeneous group of proteins comprises i) selectins, expressed in both leukocytes and EC that support leukocyte tethering and rolling, ii) leukocyte integrins and iii) members of the immunoglobulin (Ig) gene

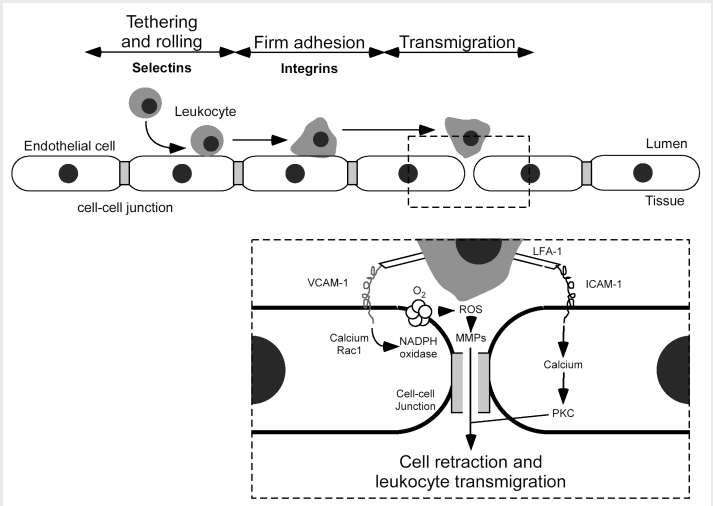

Figure 1.2 - **Leukocyte extravasation into inflamed tissues**. Leukocytes tether to ECs through binding of selectins to their ligands, an interaction permissive to continuous movement, i.e. rolling. Leukocyte rolling induces both cell surface redistribution and protein conformational changes in another class of adhesion molecules expressed in leukocytes, i.e. integrins, resulting in increased affinity for their ligands. Interaction between integrins and their ligands expressed in ECs, i.e ICAMs and VCAMs, mediates leukocyte arrest and firm adhesion. Transmigration takes place as leukocytes extend protrusions at sites of interendothelial junctions. Engagement of VCAM-1 and ICAM-1 activate signalling pathways in ECs required for leukocyte transmigration. VCAM-1 engagement stimulates endothelial NADPH oxidase activity and concomitant ROS production via calcium release and activation of the Rho-family GTPase Rac1[7]. ROS generation is required for the activity of EC-associated matrix metalloproteinase (MMP) that, in turn, are responsible for the proteolitic cleavage of proteins involved in EC junctions[10]. ICAM-1 engagement induces calcium-mediated protein kinase C (PKC) activation which results in phosphorylation of actin-associated proteins and cytoskeletal rearrangement in ECs[15]. These events ultimately result in EC retraction allowing for leukocyte transmigration.

superfamily, i.e. intercellular adhesion molecule (ICAM)-1, -2 and -3 and vascular cell adhesion molecule (VCAM)-1 expressed in the surface of EC. Interaction between these two latter sets of molecules allows the transition from rolling to firm adhesion and arrest of cells at inflamed sites (Figure 1.2). The engagement of adhesion molecules in ECs activates integrin-dependent signaling pathways leading to cytoskeleton reorganization, alteration of cell-cell junction proteins function and/or contractile forces in ECs allowing leukocytes to cross the endothelium monolayer, i.e. transmigration (Figure 1.2). Alternatively, leukocytes can also cross the endothelial monolayer via transcellular movement, where cells pass through the cellular body of an EC. The preference for one or other route of leukocyte movement across the endothelium depends on the type of leukocyte and EC, the activation state of both cell types and signalling events[31]. Ultimately, these interactions have as consequence the accumulation of polymorphonuclear leukocytes with microbicidial activity, i.e. neutrophils, latter followed by infiltration of mononuclear cells, i.e. monocytes/Mø at the inflamed site, as initiation switches into the effector phase of the inflammatory cascade.

1.1.2. Effector phase

Neutrophils are polymorphonuclear cells of the innate immune system, responsible for the phagocytosis, killing and digestion of potentially harmful microorganisms. This subpopulation of leukocytes of the innate immune system is thought to provide the first line of defence against infections (Figure 1.3). Tissue-infiltrating neutrophils trigger both oxygen-independent and -dependent effector mechanisms aimed at pathogen killing. A critical oxygen-independent mechanism is degranulation, which consists in the mobilization and fusion of cytoplasmic granules with vacuoles containing phagocytosed microbes, enriching it with microbicidial molecules. These include proteases, hydrolases and defensins responsible for the killing of pathogens either by proteolytic digestion or by interfering with the integrity of cellular membranes. Alternatively, granules can fuse with the cellular plasma membrane leading to the release of their content into the extracellular space[32]. Concurrently, the microbicidial activity of neutrophils is further enhanced by the respiratory burst, a process that involves the generation of reactive oxygen species (ROS), namely superoxide anion ($O_2^{\cdot -}$), by the nicotinamide dinucleotide phosphate (NADPH) oxidase

complex. This enzymatic system encompasses a heterodimeric catalytic core consisting of the $p22^{phox}$ and $gp91^{phox}$ subunits distributed between the plasma membrane and the membrane of cytoplasmatic granules. Phosphorylation and translocation to the membrane of the cytosolic components, i.e. $p47^{phox}$ and $p67^{phox}$ and the small GTPase Rac1, results in the assembly and activation of the complex[33]. While the amount of ROS produced in neutrophils is greater than in Mø, the latter are the major source of NO and its reaction derivatives, collectively known as reactive nitrogen species (RNS) via the iNOS pathway[34]. ROS- and RNS-generating systems have a crucial role in the resistance to microbial pathogens. Both reactive micromolecules mediate DNA damage by interfering with atomic groups with essential chemical functions in DNA stability, synthesis and protection and disturb protein stability and enzyme activation due to disruption of moiety groups. Furthermore, RNS and ROS can act synergistically, resulting in the generation of highly deleterious products such as peroxynitrite ($ONOO^-$)[35]. Finally, ROS can also influence the microbicidial activity of granule proteins by regulating the pH in the phagocytic vacuole in a way as to activate vacuolar proteases[32]. However, given their unspecific and potentially deleterious action in host cells, ROS and RNS production must be tightly regulated to avoid unfettered tissue damage in the course of pathogen killing.

During the process of clearing the noxious cause of insult, negative feedback mechanisms progressively raise the threshold for sustaining inflammation. Once these “stop” cues superimpose themselves, resolution, the third and last phase of an acute inflammatory reaction, ensues.

1.1.3. Resolution phase

Resolution of inflammatory reactions was for long considered to be a passive phenomenon, resulting from the exhaustion or degradation of pro-inflammatory mediators. The current view is that it is a highly active and regulated process aiming at restoring the inflamed tissue to its prior physiological state. Two main mechanisms operate to resolve inflammation. On one hand, there are auto-regulatory negative feedback systems that act over major signal transduction pathways involved in the onset and progression of inflammatory reactions brought about by newly synthesised mediators or “early” pro-inflammatory products. An alternative, but not mutually

exclusive, mechanism underlying the regulation of inflammatory responses relies on the elimination of leukocytes of the innate system after clearance of the inciting agent (Figure 3).

The molecular mechanisms underlying resolution of inflammatory responses are still largely unknown. As PRR activation and downstream signal transduction are crucial to drive inflammation forward, it is likely that molecules involved in the regulation of inflammatory reactions interfere with these same pathways. A prototypical example is A20, a gene whose expression is dependent on the activity of NF-κB[36]. This zinc finger protein, involved in ubiquitin editing on signal transduction molecules, has been shown to dampen TLR4, TLR2 and RIG-1 activation[37-39]. Additional negative feedback loops targeting TLR-mediated signalling include, among others, non-functional splice variants of the adaptor molecule MyD88, soluble forms of TLRs that block the interaction with other co-receptors and the ubiquitin-mediated degradation of TLRs by the E3 ubiquitin ligase TRIAD3A[40].

The turnover and timely removal of innate immune cells at the site of inflammation, especially neutrophils, is paramount for the resolution of inflammation (Figure 1.3). Apoptosis, i.e. programmed cell death, is a physiological mechanism for the non-inflammatory removal of cells that controls the extent of neutrophil-driven inflammation and tissue damage. Neutrophil apoptosis is associated with an overall decrease in cellular function revealed by diminished production of ROS, cytokines and phagocytic capacity[41]. Several molecules that dampen inflammation do so, at least partially, by promoting neutrophil apoptosis. One of such molecules is IL-10, a prototypical anti-inflammatory molecule that accelerates apoptosis of lipopolysaccharide (LPS)-activated neutrophils[42]. Moreover, endogenously produced lipid derivatives from the cycloxygenase (COX)2 and the lipoxygenase (LOX) systems are also involved in the safe disposal of neutrophils. In the early phases of inflammation, COX2 and LOX systems produce pro-inflammatory PGE_2 and leukotrienes, respectively. At later stages, however, COX2 switches to the production of anti-inflammatory PGs, namely PGD_2. Both PGD_2 and/or its dehydration metabolite of the J_2 series, i.e. 15d-PGJ_2, can induce apoptosis of inflammatory cells[43, 44] and lymphocytes[45]. At the same time, lipoxins resulting from LOX activity promote phagocytic clearance of apoptotic cells by Mø[46]. Other anti-inflammatory effects of these lipid mediators relate to the inhibition of leukocyte trafficking and activation[47]. Apoptotic neutrophils undergo cell surface alterations, resulting in the exposure of phosphatidylserine on the plasma membrane, a recognition signal for phagocytosis by infiltrating

Mø. The engulfment of apoptotic cells by Mø suppresses the release of pro-inflammatory mediators and favors the secretion of transforming growth factor (TGF)-β, which underlies to a significant extent, the anti-inflammatory function of neutrophil apoptosis and its critical role for inflammatory resolution[48].

Other regulatory loops involved in the control of inflammatory responses rely on the action of "early" pro-inflammatory mediators revealing that temporal and spatial positioning of these biochemical pathways is crucial for the productive progression and resolution of the inflammatory response. This paradox is probably best illustrated by the anti-inflammatory actions of TNF-α and IFN-γ. In the initial stages of inflammation these cytokines promote the inflammatory reaction. However, in later stages IFN-γ production by lymphocytes via TNF-α-induced release of IL-12 suppresses chemokine production by Mø, namely macrophage inflammatory protein (MIP)-1α and MIP-1β thus limiting leukocyte recruitment [49].

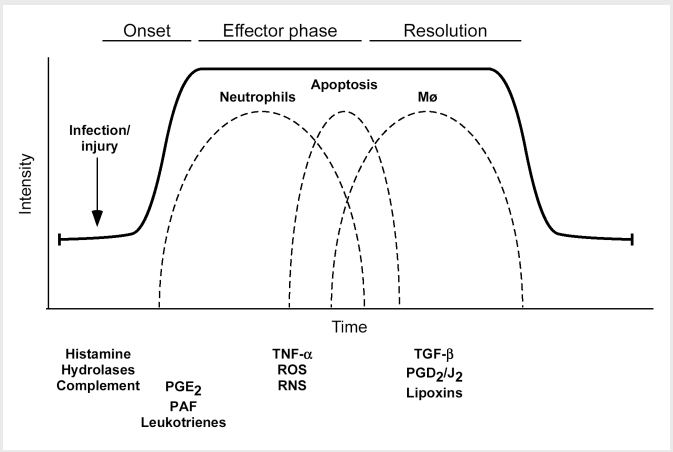

Figure 1.3 – Progression of inflammatory reactions and associated mediators. Upon injury and/or infection, tissue resident Mø and/or mast cells are activated via PRR engagement, releasing a plethora of inflammatory mediators (e.g. histamines, hydrolases, prostaglandins and leukotrienes) that trigger the initiation of inflammatory response. Neutrophils, followed by Mø, infiltrate the site of infection and/or injury where they release pro-inflammatory mediators (e.g. ROS and RNS) in a way as to deal with the noxious stimuli. The concerted action of cytokines (e.g. TNF) and pro-inflammatory mediators results in reinforcement of leukocyte infiltration and activation sustaining the inflammatory reaction and microbial clearance. Once the noxious stimulus is eliminated, neutrophils have to be disposed in a controlled and effective manner. Anti-inflammatory prostaglandins and lipoxins attenuate cell migration and promote apoptosis of neutrophils. These are engulfed by Mø, which release molecules, i.e. TGF-β, that aid at resolving the inflammatory reaction and promote the return to homeostasis.

ROS and RNS are two other sets of molecules that can contribute to the resolution of inflammation. Oxidized lipids resulting from ROS production can inhibit TLR4 signalling[50]. Furthermore, the pro-oxidative environment triggered by these reactive micromolecules is responsible for the induced expression of so-called "stress responsive genes", a set of genes crucial to limit inflammation. This group

includes Heme Oxygenase-1 (HO-1), a major topic in this thesis that shall be addressed in a separate section (*see Chapter 1, section 2*).

1.2. Adaptive immune responses

There are circumstances where innate immunity is not able to eliminate infection and requires the induction of the adaptive immune response. Adaptive immune responses rely on B and T cells that display somatically generated receptors on their surface, i.e. B cell receptor (BCR) and T cell receptor (TCR), responsible for the recognition of epitopes, consisting in specific three-dimensional features or linear peptide sequences, respectively, within antigens.

During the course of an inflammatory reaction, PRRs orchestrate not only the recruitment and sustained activation of leukocytes at the site of infection but also assume a critical role in promoting the initiation of adaptive immunity. This occurs via the activation and maturation of DCs, the only APCs capable of activating naïve T_H lymphocytes. This process involves antigen capture at the site of infection, migration into the secondary lymphoid organs, up-regulation of major histocompatibility complex (MHC) and costimulatory molecules (CD40, CD80 and CD86) as well as secretion of cytokines and chemokines. These events are regulated through the recognition of DAMPs via PRRs[51]. DC activation and maturation will ultimately lead to the activation and clonal

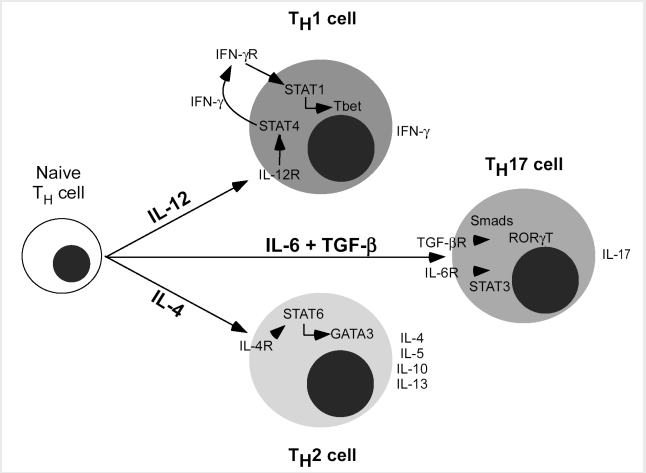

Figure 1.4 - **Differentiation of effector CD4⁺ T_H cell subsets**. IL-12 promotes the differentiation of naïve CD4⁺ T_H cells into a T_H1 phenotype. IL-12-mediated activation of signal transducer and activator of transcription (STAT)4 results in INF-γ production that, in turn, activates STAT1 and the expression of T-box expressed in T cells (T-bet), a transcription factor crucial for T_H1 development[3]. Conversely, IL-4 directs naïve CD4⁺ T_H cell towards T_H2 polarization via the activation of STAT6 and consequent expression of the GATA3 transcription factor[3]. Skewing towards a T_H17 phenotype develops in response to IL-6 and TGF-β. IL-6-dependent signalling results in STAT3 activation and induction of the transcription factor retinoid-related orphan receptor (ROR)γt upon stimulation of CD4⁺ T_H cells[11].

expansion of antigen-specific $CD4^+$ T lymphocytes. In addition, DCs also promote naïve $CD4^+$ T cells differentiation into T helper type 1 (T_H1), T_H2 or T_H17 cells (Figure 1.4) as well as to the activation, differentiation and clonal expansion of cytotoxic $CD8^+$ T lymphocyte effectors. The differentiation of naïve $CD4^+$ T_H lymphocytes into functionally distinct subsets is influenced by the density of antigen presented, the type of costimulatory molecules expressed and the cytokines secreted by the DCs[52], resulting in cells with distinct functional abilities and underlying molecular programs. T_H1 lymphocytes act by fostering cell-mediated immunity via IFN-γ production and are required for memory $CD8^+$ T cell responses. Conversely, IL-4 and IL-10-producing T_H2 lymphocytes are the most effective activators of B lymphocytes into antibody-secreting cells and thus mediate humoral-type immunity. The primary function of T_H17 cells is likely to involve host protection against extracellular bacteria although an important role in the establishment of autoimmunity has been attributed to these cells (*see Chapter 1 section 3.1.7.*). Each T_H subset negatively regulates the others through opposing effects of their respective cytokines. IFN-γ inhibits T_H2 and T_H17 skewing and promotes T_H1 differentiation. Conversely, IL-4 directs T_H2 differentiation and inhibits the development of IFN-γ-producing cells. IL-10 interferes with antigen presentation in a way as to preclude T_H1 skewing. Similarly, both T_H1 and T_H2 cytokines afford potent inhibition of T_H cell differentiation towards a T_H17 effector phenotype.

The existence of highly specific T_H cell effector responses against pathogenic microorganisms has come with a cost. The random character of gene rearrangement responsible for the generation of the BCR and TCR repertoire, i.e. the collection of antigen specificities exhibited by all these receptors expressed in a given individual, results inevitably in the generation of reactivity against self. Efficient mechanisms have evolved to preclude uncontrolled and sustained tissue destruction and/or impaired function by self-reactive B and T cells, collectively known as self-tolerance. Mechanisms regulating B cell tolerance are beyond the scope of this thesis and shall not be addressed. The following section will focus on regulatory mechanisms aimed at enforcing self-tolerance in what concerns self-reactive T cells.

1.2.1. Self-reactivity *v.* tolerance

Estimates of all TCR specificities range from 10^6 in mice[53] to 10^7 in humans[54]. Among these, TCRs that recognize self-antigens are potentially deleterious to the host and must be specifically eliminated or prevented from reacting while maintaining those that confer protection against infection, i.e. reactive against non-self. The majority of TCR specificities are "screened" during late fetal and early pos-natal life in central lymphoid organs, i.e. the thymus, in a process named central tolerance. During lymphocyte differentiation in the thymus, a vast proportion of thymocytes with no specificity for host MHC molecules fails to receive survival signals through TCR engagement and, as such, die by neglect. This results in the elimination of those T cells that, upon maturation, would not recognize any antigen/self MHC complex. The remaining T cell precursors, which are positively selected by TCR engagement, result in the generation of a highly diverse repertoire of T cells capable of recognizing antigens in a self-MHC-restricted manner. Among these there is a significant proportion carrying TCRs with high affinity for self-antigens/self-MHC complexes. If recognition of such self-antigens occurs in the thymus, developing self-reactive T cells are eliminated by apoptosis[55], guaranteeing that highly self-reactive lymphocytes are purged from the peripheral repertoire of naive T_H cells. Nevertheless, T cells with reactivity to self-antigens are found in the peripheral repertoire of healthy individuals. Several hypotheses have been put forward to explain the failure to eliminate these lymphocytes in the thymus. First, the plasticity of T cell antigen recognition makes it possible that T cells exhibiting high affinity to non-self antigens can, nonetheless, exhibit comparatively low affinity to self-antigens[56] (*see Chapter 1, section 3.1.4.*). Second, self-antigens presented in the periphery might differ or even be absent from the collection of self-peptides expressed in the thymus resulting in activation of those clones that were not negatively selected. An example is the differential expression of proteolipid protein (PLP) isoforms in the thymus and the central nervous system (CNS). While full-length PLP is expressed predominantly in CNS, a truncated version, where a major encephalitogenic determinant consisting of animoacids 139 to 145 is absent, is the main isoform present in lymphoid organs[57]. The unavailability of such an epitope for thymic negative selection results in increased frequency of anti-PLP self-reactive T cells in the peripheral compartment of naïve mice[58]. The third hypothesis reflects the critical role of MHC in shaping T cell repertoire and its association with autoimmune

diseases (*see Chapter 1, section 3.1.3.*). Inefficient presentation of thymic peptides and thus ineffective negative selection by certain disease-associated MHC alleles is thought to bias the mature peripheral lymphocyte repertoire towards autoimmunity[59, 60].

As previously discussed, negative selection, although contributing critically to the elimination of high affinity self-reactive T cells, fails to eliminate those T cells that bear a low affinity TCR for self-antigens. Under homeostatic conditions these self-reactive T cells are maintained in an inactive state suggesting the existence of additional immunoregulatory mechanisms, i.e. peripheral tolerance.

A critical mechanism via which self-reactive T_H cells are maintained under check in peripheral lymphoid organs and tissues seems to rely on the presence of regulatory T cells. From the several populations of regulatory T cells crucial for the establishment of self-tolerance and immune homeostasis, the best characterized are the naturally occurring regulatory $CD4^+$ T cells (T_{regs}). These express both the IL-2 receptor α chain (CD25) and the transcription factor forkhead box p (Foxp)3. Foxp3, whose expression is mainly restricted to the $CD4^+CD25^+$ subset of naturally occurring T_{regs}, plays a crucial role in T_{reg} development and function[61, 62]. The current view is that $CD4^+CD25^+T_{regs}$ are generated in the thymus in a process that depends on the interaction between TCR and peptide/self-MHC class II complexes. Developing thymocytes with intermediate TCR avidities to self-peptide/self-MHC class II complexes, i.e. between those that allows for positive selection and those that dictate thymic deletion, preferentially express Foxp3 and commit to the T_{reg} lineage[63]. This, together with the fact that $CD4^+CD25^+T_{regs}$ are more resistant to negative selection than T_H cells[63] is thought to bias T_{reg} repertoire towards self-reactivity. The mechanisms underlying the ability of T_{reg} to suppress self-reactive T_H responses are poorly understood but seem to depend on TCR stimulation. The fact that $CD4^+$ T cells with regulatory functions are selected based on their reactivity to self might result in an imbalance in the frequencies of T_{reg} and self-reactive T_H cells thus promoting tolerance. Moreover, several molecules and/or signal transduction pathways have been identified as mediating the suppressive effects of $CD4^+CD25^+T_{reg}$. Inhibition of IL-2 signalling, a vital factor for T_H cell proliferation and survival, might explain T_{regs} inhibitory effects, at least in *in vitro* settings[64]. By expressing the high-affinity receptor for IL-2, regulatory T cells can potentially limit IL-2 availability and impair IL-2-dependent signalling in T_H cells[65]. Despite conflicting data,

other molecules such as IL-10, TGF-β, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and its ligands CD80 and CD86 have all been proposed to be essential for T_{reg} function[66].

Alternatively, other mechanism that precludes peripheral self-reactive T_H cells from causing tissue damage and disease relies on their suppression via the induction of anergy, i.e. functional unresponsiveness of self-reactive T_H cells. Several mechanisms involving distinct molecules and signal transduction pathways in both T cells and APCs can lead to the establishment of anergy. In what concerns molecules expressed in T cells, these include but are not restricted to CTLA-4. This receptor provides inhibitory signals upon interaction with the co-stimulation molecules CD80/86 expressed in APCs[67]. In a similar manner, programmed cell death 1 (PD-1) is another receptor for the CD80/86 family members whose activation negatively modulates T cell proliferation and cytokine production[68].

DCs are major contributors in promoting peripheral tolerance. The first and most widely accepted concept of DC-mediated tolerance evolved from the two-signal theory of T cell activation[69] (Figure 1.5 and below). The notion that APCs, and in particular DCs, are capable of integrating a variety of signals in a way as to orchestrate an adaptive immune response or T cell tolerance led to the proposition by Charles Janeway of the Infectious Non-Self Theory[70]. This model is an expansion of the self/non-self discrimination principles for lymphocytes, though the focus is on innate immune system. It is based on two postulates. The first states that, under homeostatic conditions, APCs are not

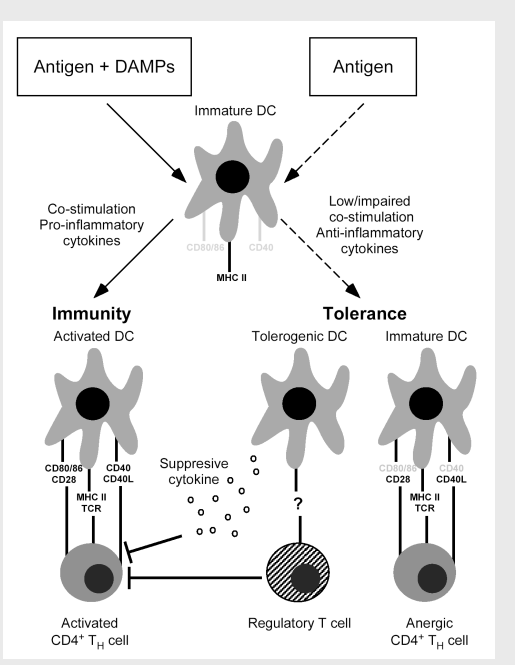

Figure 1.5 - Regulation of immune responses by DCs. During infection, the presence of DAMPs triggers phenotypic alterations via up-regulation of co-stimulatory molecules that ultimately result in the activation of T_H cells and the initiation of the immune response. In a homeostatic situation, DCs sample self-proteins resulting from cellular turnover in tissues and present them to T cells after migration into the secondary lymphoid organs. The constant trafficking and presentation of self-antigens by non-activated DCs, i.e. unable to provide the second signal provided by co-stimulatory molecules, results in anergy of self-reactive T cell clones. Other tolerogenic actions of DCs involve the release of immunosuppressive cytokines and/or induction of regulatory T cell differentiation and/or expansion[5].

competent in stimulating T cells. The second predicted that APCs express receptors for the recognition of molecular patterns exhibited by infectious microorganisms in a way as to provide the required co-stimulatory signals for effective T cell activation and function. The discovery of PRRs and their ligands (*see Chapter 1, section 1.1.1.*) has provided the molecular basis for the Infectious Non-Self Theory. However, the assumption that PRRs allow for specificity of innate cells based on self/nonself discrimination has been challenged by the suggestion that endogenous, host-derived ligands can elicit PRR activation and promote immune responses (Figure 1.1). Such signals can be generated when cells undergo necrosis, i.e. pathologic cell death, an event associated with the release of intracellular molecules in the extracellular milieu. Such is the case of members of the heat shock protein (HSPs) family. HSPs participate in protein folding and assembly and their synthesis is increased under stress conditions. Until now, HSP60, 70 and 90 have been suggested to act as TLR2 and TLR4 ligands. Host chromatin and endogenous messenger (m)RNA have also been suggested to act as DAMPs via TLR9 and 3, respectively. Uric acid crystals generated by damaged cells have also been shown to engage NALP3 and trigger inflammation[71]. Notwithstanding, the identification of endogenous molecules capable of eliciting PRR signalling has to be taken with caution given the possibility of contamination with LPS or other pathogen-derived PRR agonists. The Danger Theory was proposed in order to accommodate observations regarding the role of these endogenously-driven molecules in eliciting PRR signalling[72]. According to Polly Matzinger, "(…) *the immune system is more concerned with damage than with foreignness* (…)"[73]. In the Danger Theory, target tissue itself dictates the shaping of the adaptive immune response as only pathogens responsible for tissue damage, and hence releasing danger signals, would elicit an active response. Autoimmunity would therefore arise as a consequence of self-antigen presentation in a context where danger signals are present. The apparent association between infection and the induction of autoimmune diseases (*see Chapter 1, section 3.1.4.*) supports, at least to same extent, this hypothesis. The notion that target tissue contributes to the progression of immune responses would also suggest that genes that limit tissue damage, i.e. protective genes, can potentially modulate such responses by limiting the generation of endogenous DAMPs.

2. Protective genes

The concept of protective genes was first proposed in the context of transplantation upon recognition that, apart from the recipient's immune response, graft survival is also dictated by its ability to protect itself from injury. The molecular basis for these protective mechanisms relies on a set of genes that, when expressed in the grafted tissue, exert strong anti-inflammatory and cytoprotective effects[74, 75]. One of those genes is Heme Oxygenase-1, whose pleiotropic effects in the control of inflammatory and immune reactions will be the main focus of the next section.

2.1. Heme Oxygenase-1 (HO-1)

Tenhunen and co-workers were the first to ascertain that the rate limiting step in the oxidative cleavage of heme into biliverdin (BV), carbon monoxide (CO) and free Fe, required the action of a microsomal enzymatic complex present in splenic Mø[12, 76]. The enzyme responsible for the catalytic activity was subsequently identified as being heme oxygenase[77]. The heme oxygenase system has a broad distribution not only in vertebrates but also in invertebrates, higher plants, algae, fungi and bacteria, although its function varies according to the needs of individual species.

There are two heme oxygenase isoforms, namely heme oxygenase-1 (HO-1; 32 kDa) and -2 (HO-2, 36 kDa), each encoded by a distinct gene, i.e *Hmox-1*/*HMOX-1* and *Hmox-2*/*HMOX-2* in mice and humans, respectively[78]. A third isoform, HO-3, is though to be a pseudogene derived from that of HO-2 based on the inability to detect HO-3 mRNA and/or protein [78] and shall not be further addressed.

The protein sequence homology between HO-1 and HO-2 is only 43%. However, both enzymes in rat, mouse and human share a highly conserved "heme pocket" consisting of a 24 aa segment containing a proximal heme-binding histidine (His) residue, i.e. His-25 in human HO-1 and His-45 in human HO-2[79]. In addition, the "heme pocket" in both enzymes is flanked by regions that, although not sequence-conserved, have highly conserved secondary structures[80]. Despite their conserved structure of the catalytic site and similar molecular mechanisms of enzymatic activity, HO-1 and HO-2 differ significantly in their tissue expression patterns as well as in their inducibility of expression in different cell types. HO-2 is constitutively expressed and is the predominant isoform in a restrained set of tissues, namely the brain[81] and testis[82]. Expression of HO-2 at lower

levels has also been reported in other tissues[79]. The stable protein levels of HO-2 along with the observations that this enzyme acts as a potassium channel-associated oxygen sensor[83] and that HO-2-null mice (*Hmox*-2$^{-/-}$) have increased susceptibility to hyperoxic injury[84] sustain the view of a regulatory role for HO-2 in oxygen homeostasis. Furthermore, it has been postulated that HO-2 might counteract the oxidative neurotoxicity associated with Alzheimer disease[85]. In clear contrast with the role of HO-2 in homeostasis, HO-1 is highly inducible by a plethora of inducing agents under pathophysiologic conditions, underlying the central role of this enzyme in pathophysiologic conditions.

2.2. Heme biology

Heme, the substrate of HO activity, is a tetrapyrrole molecule consisting of a protoporphyrin IX ring with a central Fe ion (Fe^{2+}) generated via a metabolic pathway that includes both mitochondria- and cytosolic-associated enzymes[86]. δ-aminolevulinic acid synthase (ALAS), the rate-limiting enzyme in heme biosynthesis, catalyses the formation of δ-aminolevulinic acid from glycine and succinyl coenzyme (Co)A[87]. There are two ALAS isoforms: a developmentally regulated isozyme exclusively expressed in cells of the erythroid lineage (ALAS2) and a non-specific housekeeping isoform, ALAS1, expressed primarily in liver but also present in other tissues[88]. In non-erythroid cells, heme biosynthesis is regulated by ALAS1 activity in response to intracellular heme concentration[89]. Such is achieved mainly by heme-mediated inhibition of ALAS1 synthesis[90] and subsequent transport into the mitochondria matrix where it is post-transcriptionally processed into the mature enzyme form[86].

Heme serves as a prosthetic group in numerous hemoproteins. Under physiological conditions, heme is essential for various cellular mechanisms that include the transport of reversibly bound oxygen in the heme-containing hemoglobin and short-term storage of oxygen in myoglobin as an additional source for oxidative capacity during muscle contraction. Heme also plays a central role in the generation of cellular energy and detoxification given that it is a structural component of redox cytochromes from the respiratory chain in mitochondria, and of the P450 class of detoxifying cytochromes responsible for the oxidation of hydrophobic to water-soluble compounds. Other hemeproteins include NADPH oxidase, those involved in antioxidant defences

such as catalase and glutathione peroxidase as well as enzymes involved in signal transduction processes such as nitric oxide synthases and guanylate cyclase (GC).

Apart from its role as prosthetic group, augmented concentrations of non-protein bound heme can be highly deleterious to cells. During Earth's evolutionary history, the appearance of microorganisms capable of producing energy from carbon dioxide (CO_2), water and sunlight operated a significant alteration in atmosphere composition from a sparse to the current oxygen (O_2)-abundant state. Aerobic organisms depend on this photosynthetically generated O_2 to produce biochemical energy in a process termed respiration. This process involves the oxidation of energy-rich molecules, usually accomplished using O_2 as an electron acceptor, and the subsequent synthesis of small molecular mediators that act as chemical energy storage, i.e. adenosine tri-phosphate (ATP). However, this acquired oxygen dependency poses a paradox, as acceptance of unpaired electrons by O_2 leads to the formation of potentially deleterious ROS (Figure 1.6). As a consequence, cells have developed several mechanisms to insure that, under homeostatic conditions, ROS levels are kept to a minimum (Figure 1.6). Concurrently with the action of several enzymes, proper Fe compartmentalization within the cell is yet another mechanism to limit ROS formation (Figure 1.6). The potentially hazardous effects of free heme is that it is highly hydrophobic[91] and is one of the most abundant sources of redox-active Fe. A critical feature of Fe-dependent damage to cells is the permeation of the metal into the hydrophobic milieu of intact cells[92] such as accomplished through heme-mediated trafficking across plasma membranes.

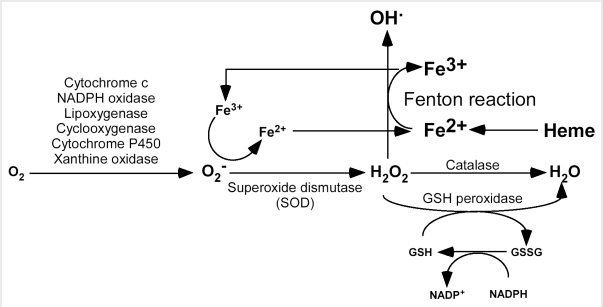

Figure 1.6 - **Pathways of ROS generation**. ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxil radical ($OH\cdot$), are generated in cells by several pathways. O_2^- is constantly generated in the mitochondria by aerobic cell metabolism. Furthermore, O_2^- can also be produced by NADPH oxidase, lipoxygenase, cyclooxygenase, NADPH cytochrome P450 reductase and hypoxanthine/xanthine oxidase. Under homeostatic conditions, ROS accumulation is limited by several enzymatic systems. Superoxide dismutase (SOD) converts O_2^- into H_2O_2, which is subsequently converted to H_2O by catalase and glutathione (GSH) peroxidase. In the presence of H_2O_2, the Fe contained within heme can catalyse the formation of highly reactive radical $OH\cdot$ via the Fenton reaction. Autoxidized Fe^{3+} is reduced back by O_2^- to the Fe^{2+} form, which subsequently catalyzes the decomposition of H_2O_2.

Red blood cells (RBCs), which are vulnerable to lysis, constitute, by far, the most abundant source of heme, found as the prosthetic moiety of hemoglobin. Under normal conditions, when low

plasma hemoglobin levels are produced, such as during nuclear extrusion of erythroblasts, hemoglobin dissociates into αβ dimmers that bind to haptoglobin (Hp) and is internalised via interaction with the cell surface receptor CD163 in order to be metabolised.

Unbound plasma hemoglobin is rapidly oxidized into ferrihemoglobin, also called methemoglobin, which, in turn, dissociates into globin and ferriheme (Fe^{3+}). Ferriheme can, at this stage, be bound by hemopexin (Hx), the plasma protein with the highest binding affinity for heme. Following interaction with heme, Hx undergoes a conformational change that enables the interaction with its specific receptor, internalisation of the heme-Hx complex and, ultimately, heme degradation. The important role of Hp and Hx in counteracting the deleterious effects of hemoglobin and heme, respectively, is corroborated by the observations that both *Hp*- and *Hx*-null mice suffer from oxidative stress-mediated renal injury after haemolytic stimulus[93, 94].

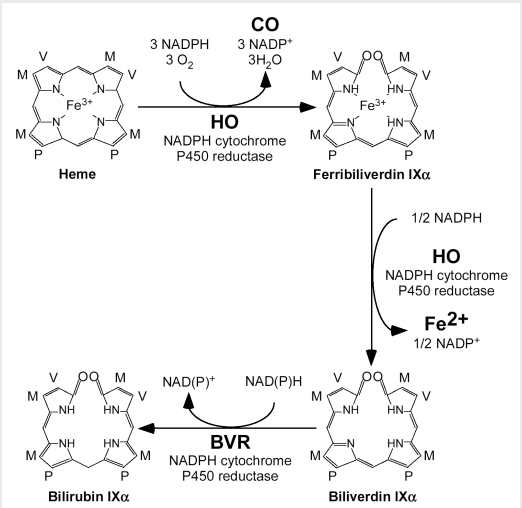

Figure 1.7 - **Heme degradation**. Heme is degraded by both HO-1 and HO-2. The reaction requires three oxidative steps with the consumption of three oxygen (O_2) molecules and seven electrons provided by NAD(P)H-cytochrome-P450-reductase. There is an initial oxidation resulting in the reduction of the heme-bound ferric Fe (Fe^{3+}), which binds O_2. In each reaction step, the Fe-bound O_2 accepts a second electron and attacks the heme ring. The resulting reactive oxygen intermediate hydroxylates the pyrrole ring at the α-methene bridge carbon resulting in its elimination as CO. The subsequent NADPH–cytochrome-P450-reductase and O_2 dependent reduction results in the formation of ferribiliverdin IXα, which is further reduced to the ferrous state by NAD(P)H-cytochrome-P450-reductase, resulting in the release of the free Fe (Fe^{2+}) followed by dissociation of biliverdin IXα from the HO-1 protein[6]. The reduction at the central C-10 carbon by the NAD(P)H-cytochrome-P450-reductase-dependent biliverdin reductase results in the conversion of biliverdin IXα in bilirubin IXα[12, 13].

Under pathophysiologic conditions associated with extensive RBC lysis and/or tissue destruction, the Hp and Hx systems might become saturated resulting in increased concentrations of free heme. This can account for the intracellular mobilization of redox-active Fe, thus contributing to Fe homeostasis disturbance and the synergistic amplification of cytotoxic effects arising from activated oxygen (Figure 1.6). This mechanism was first illustrated by the observation that free heme can sensitize ECs to H_2O_2-mediated toxicity[95]. Such is accomplished via a ROS-mediated process that probably

involves: i) membrane lipid peroxidation[96] leading to cellular membrane disruption; ii) peroxidation of cytosolic proteins responsible for the formation of non-reducible covalent cross-linkages[97] or protein fragmentation[98] and iii) DNA strand scission with the consecutive degradation to oligonucleotides[99].

2.3. Heme catabolism and control of inflammation

The salutary effects associated with the expression of HO-1 seem to depend mainly on its enzymatic activity leading to BV generation accompanied by the release of heme Fe in the ferrous form (Fe^{2+}) and CO (Figure 1.7). That HO-1 acts in a protective manner is suggested by the observation that *Hmox-1/HMOX-1* gene disruption in mice or humans is associated with more or less severe forms of chronic inflammation. In keeping with this notion, *Hmox1*$^{-/-}$ mice have non-mendelian segregation, growth retardation, lymphadenopathy, leukocytosis, splenomegaly, Fe deposition in tissues and glomerulonephritis[100, 101]. Furthermore, these mice are highly sensitive to oxidative damage and have increased lethality following LPS administration[101]. Similarly, HO-1-deficiency in one patient was associated with growth hindrance, anaemia, Fe deposition and leukocytosis as well as renal damage with subsequent proteinuria and hematuria[102]. The involvement of HO-1 in controlling inflammation is further corroborated by the association between polymorphism in the *HMOX-1* promoter and a growing list of diseases with underlying inflammatory mechanisms (Table 1.1). The most extensively studied variation present in the HO-1 gene promoter region, located approximately in the –200 position, consists of a length polymorphism of varying dinucleotide repeats $(GT)_n$. Alternating purine-pyrimidine sequences such as the ones present in the *HMOX-1* promoter mediate the formation of DNA structures capable of negatively regulating gene transcription[103]. Although the number of dinucleotide repeats ranges from $(GT)_{10}$ to $(GT)_{40,}$ the distribution is bimodal with the most represented alleles exhibiting 23 and 30 repeats. Alleles with repeat lengths lower then 25, classified as small (S), are correlated with increased transcriptional activity and HO-1 inducibility in response to several stimuli when compared to large (L) alleles, i.e. more that 25 repeats[104, 105]. In addition to the $(GT)_n$ polymorphism, there is a single nucleotide polymorphism (SNP) at position –413 (A or T)[106]. However, the mechanism by which this polymorphism regulates gene transcription remains elusive.

Disease	Polymorphism
Pulmonary diseases	
Emphysema in smokers	$(GT)_n$
Chronic obstructive pulmonary disease	$(GT)_n$
Pneumonia in elderly	$(GT)_n$
Lung adenocarcinoma in smokers	$(GT)_n$
Cardiovascular diseases	
Hypertension in women	T(-413)A
Coronary artery disease	T(-413)A
Coronary artery disease in patients with risk factors	$(GT)_n$
Coronary artery disease in type II diabetic patients	$(GT)_n$
Abdominal aortic aneurysms	$(GT)_n$
Restenosis after coronary stenting	$(GT)_n$
Restenosis after peripheral angioplasty	$(GT)_n$
Coronary adverse effects in peripheral artery disease	$(GT)_n$
Renal diseases	
Kidney allograft function	$(GT)_n$
Other disease entities	
Idiopathic recurrent miscarriage	$(GT)_n$
Susceptibility to apoptosis	$(GT)_n$
Arteriovenous fistula failure in haemodialysis patients	$(GT)_n$

Table 1.1 – Disorders associated with *HMOX-1* promoter polymorphism. Adapted from[4].

The molecular basis for the protective effect of HO-1 is complex and probably multifactorial. Limiting the availability of heme *per se* is likely to contribute to the salutary effects of HO-1 by precluding its pro-oxidant activity. Heme-mediated oxidative damage is thought to be involved in the pathogenesis of acute kidney disease associated with rhabdomyolysis and intravascular hemolysis due to mitochondrial dysfunction[107]. An involvement of heme has also been shown in the establishment and progression of atherosclerosis by acting as a catalyst for the oxidation of low-density lipoprotein which, in turn, is a mediator of the disease[108]. Moreover, heme mediates the development of cerebral malaria (CM) in mice (*see Chapter 4, section 3.5.*). Limiting free heme availability might also impact on the activity of proteins where heme functions as the prosthetic group. These include the pro-inflammatory enzymes COX2 and NADPH oxidase (*see Chapter 1, sections 1.1.1. and 1.1.2.*). Increased levels of HO-1 are associated with decreased COX2

expression/activity[109] as well as with decreased production of ROS by down-modulating the expression of gp91^{phox}, the heme-containing catalytic subunit of the NADPH oxidase complex[110].

A growing body of evidence supports the notion that downstream products of HO-1 action on heme, i.e. BV, CO and free Fe (Figure 1.7), are central in affording its protective effects. These products have been shown to have immunomodulatory, anti-oxidant and anti-apoptotic properties. Despite the apparent functional overlap of the different products resulting from heme catabolism, they might cooperate to afford protection by impacting on different cellular functions/mechanisms[111].

2.3.1. Carbon monoxide (CO)

CO was regarded for a long time solely as a toxic gas based on its strong affinity to hemoglobin and thus to cause tissue hypoxia. However, in the last decade the role of CO as a signalling mediator with anti-inflammatory, cytoprotective and vasodilator properties has been revealed in several experimental systems.

CO modulates acute and chronic inflammation by impacting on crucial cellular components of both the innate and adaptive immune systems. It controls the production of both pro- and anti-inflammatory mediators by innate immune cells. When exposed to CO, PRR-activated Mø decrease the expression of TNF-α, IL-1β and MIP-1β while enhancing that of the anti-inflammatory cytokine IL-10[112]. A somewhat similar mechanism has been identified in DCs, where pharmacological induction of HO-1 has been shown to decrease the levels of IL-12p40, TNF-α and IL-6 production while not affecting those of IL-10[113]. Moreover, HO-1 expression is induced in Mø exposed to IL-10 while pharmacological inhibition of HO activity and/or CO scavenging precluded the anti-inflammatory effects of IL-10 in activated Mø[114]. These observations suggest that the interplay between IL-10 and HO-1 constitute a positive-feedback mechanism aiming at amplifying the anti-inflammatory response mediated via IL-10 and probably by other anti-inflammatory molecules as well. The mechanisms underlying the ability of CO to modulate the molecular machinery of the cell have been proposed to involve the generation of a transient burst of ROS[115-117]. CO inhibits the mitochondrial electron transport chain complex IV, i.e. cytochrome *c* oxidase, resulting in mitochondrial ROS production[118]. The shift in cellular redox state activates transcription

factors with a downmodulatory role in inflammation such as peroxisome proliferator-activated receptor (PPAR)γ and hypoxia-inducible factor (HIF)-1α[116, 117]. In addition from modulating pro-inflammatory cytokine production, pharmacological HO-1 induction and/or exposure to CO functionally modulate antigen presentation by APCs. *In vitro* induction of HO-1 renders DCs refractory to LPS-mediated maturation as assessed by expression of MHC class II and the co-stimulatory molecules CD80 and CD86[113] an effect mediated, at least partially, by CO (*see Chapter 2, section 3.5.*). The modulation of specific TLR signalling pathways by CO has provided a molecular basis for this effect. Upon ligand recognition, TLR receptors and adapter molecules are recruited to lipid rafts via a mechanism that is dependent on the generation of ROS[119, 120]. By targeting the heme moiety of the NADPH oxidase $gp91^{phox}$, CO decreases ROS production and impairs TLR trafficking, thus inhibiting TLR-dependent signalling[120]. Inhibition of $gp91^{phox}$ activity by CO constitutes an example of a broader concept where CO can potentially modulate the activity of any protein where heme functions as a prosthetic group by acting as a physiological ligand. iNOS, another heme-containing protein with a critical role in inflammation (*see Chapter 1, sections 1.1.1. and 1.1.2.*) can also be functionally targeted by CO[121].

Besides targeting directly the function of inflammatory cells, CO exerts other protective effects that can modulate the outcome of inflammation. During inflammatory reactions, oxidative stress and/or cytotoxic cytokines can contribute to exacerbate inflammation by promoting tissue damage. It is possible, therefore, that the cytoprotection afforded by CO, namely in ECs[122], might contribute to its overall anti-inflammatory properties. The cytoprotective effect of CO requires the activation of the p38 family of MAPK, specifically that of the anti-apoptotic p38β, while down modulating the levels of the pro-apoptotic p38α isoform[123]. In addition, CO is cytoprotective in hepatocytes[124] and fibroblasts[125], an effect that might restrict the generation of endogenous-derived DAMPs, thus limiting the activation of innate immune cells and concomitantly that of T_H lymphocytes. CO has also been suggested to hamper proliferation of human T cells via inhibition of IL-2 production[126]

2.3.2. Biliverdin (BV) and bilirubin (BR)

Stocker and colleagues were the first to propose a potential physiologic role for BV/bilirubin (BR) after the observation that both bile products can act as potent antioxidants[127, 128], providing the basis for the antioxidant function of HO-1. BR scavenges the highly deleterious radical ·OH (*see Chapter 1, section 2.2.*) in a more effective way than α-tocopherol (vitamin E), one of the most powerful antioxidants[127]. The ROS scavenging properties of BV/BR extend to other reactive species, namely those derived from NO, such as $ONOO^-$[129]. The antioxidant effects of BR, shown to protect cells from 10^4-fold higher concentrations of H_2O_2, cannot be accounted for by stoichiometric scavenging activity. Instead, a redox cycle where each molecule of BR is readily oxidized to BV and reduced back to bilirubin by biliverdin reductase (BVR) restores the intracellular pool of BR available for reactive species scavenging[130]. In addition, BR impacts directly on the generation of ROS by interfering with the redistribution of the $p47^{phox}$ subunit of the NADPH oxidase complex[131]. Presumably based on their role as ROS scavengers, BR/BV can mediate the beneficial effects of HO-1 induction in several cell types and pathologies by affording potent antioxidant protection.

Population studies have demonstrated an inverse relationship between serum BR levels and incidence and/or progression of coronary artery disease or atherosclerosis[132, 133]. Furthermore, in individuals afflicted with Gilbert syndrome, where a deficiency in uridine diphosphate glucuronyltransferase (UDPGT) results in hyperbilirubinemia, there is a 6-fold decrease in prevalence of ischemic heart disease when compared to normal populations[134]. The beneficial effects of BV/BR in the context of atherosclerosis-related diseases are associated, to a great extent, to their ability to interfere with the cell cycle machinery and suppress the proliferation of SMCs, thus inhibiting vascular lumen narrowing[135]. Such observations underlie the ability of BV/BR to function as modulators of cell signalling pathways. In fact, BV/BR have been shown to regulate the function of several protein kinases, namely protein kinase A and C[136]. Given the key role of protein phosphorylation in cellular regulation, it is possible that the bilirubin-mediated inhibition of protein kinases might contribute to its overall biologic effects.

Anti-inflammatory effects of BV/BR have also been described. Leukocyte rolling and adhesion is a key factor in the progression of an inflammatory response (*see Chapter 1, section*

1.1.2.). Pharmacological induction of HO-1 inhibits to a large extent leukocyte rolling and adhesion over ECs *in vivo*, and effect mimicked by BR but not CO[137]. These findings are in accordance with the *in vitro* observation that BR suppresses the expression of adhesion molecules, namely E-selectin and VCAM-1, in stimulated ECs where CO does not[138].

2.3.3. Iron (Fe)

Heme catabolism by HO-1 generates free Fe, a potent catalyst in the formation of ROS (*see Chapter 1, section 2.2.*). The cytoprotection afforded by HO-1 is related to its capacity to finely regulate intracellular Fe pools via several mechanisms. One of such strategies relies on the activation of an ATPase pump that actively transports free Fe out of the cell[139, 140]. Ferritin, which sequesters intracellular free Fe, constitutes another system involved in the regulation of Fe content[141, 142]. This multimeric protein, composed of heavy (H) and light (L) chains, is the major reservoir of non-metabolic Fe in cells storing up to 4000 ions of Fe per molecule of ferritin. Along with its Fe sequestration potential, the H-ferritin subunit exhibits ferroxidase activity, i.e. catalyzes the oxidation of Fe^{2+} ions to the less reactive ferric form[143]. The Fe released from heme catabolism by HO-1 induces the expression of H-ferritin at the translation level[144]. Several observations suggest that H-ferritin can recapitulate the cytoprotective actions of HO-1. In fact, induction of ferritin has been proposed to be as protective as that of HO-1[141]. Adenovirus-mediated overexpression of H-ferritin is anti-apoptotic in hepatocytes and ECs[145]. Notwithstanding, in other pathologic conditions the protective effects of HO-1 do not seem to depend on ferritin expression[146].

The molecular mechanisms underlying the cytoprotective effect of H-ferritin are best elucidated in the context of TNF-α-mediated apoptosis. During inflammatory reactions, TNF-α signaling via TNF-R results in activation of NF-κB dependent transcription thus coordinating changes in gene expression crucial for inflammation and immunity. Paradoxically, when NF-κB activity is blocked, this pleiotropic cytokine also triggers cell death by apoptosis. This occurs via a mechanism that involves the accumulation of intracellular ROS leading to the sustained JNK activation, involved in pro-apoptotic pathways[147]. However, concomitant to ROS accumulation, H-ferritin expression is increased via NF-κB-dependent transcriptional activity. The Fe-chelating

capacity of H-ferritin devoids Fe from promoting the generation of ROS and impairs activation of the JNK pathway[148]. This effect accounts for the potent anti-apoptotic effects of H-ferritin and probably contributes in a significant manner to the cytoprotective effect of HO-1 as well.

3. Inflammatory mechanisms in the pathogenesis of disease

The aforementioned mechanisms (*see Chapter 1, sections 1.1.3. and 2.*) allow for the desirable self-limiting and self-resolving character of an inflammatory reaction. However, deregulated amplification pathways can result in acute inflammatory conditions while failure in resolution mechanisms will dictate a chronic inflammatory state associated with the development of a vast number of diseases with apparently unrelated pathophysiologic mechanisms. Table 1.2 lists conditions where inflammation is thought to contribute to disease establishment and/or progression.

Disorders in which an important pathogenic role is assigned to inflammation		
Alzheimer's Disease	Hashimoto's thyroiditis	Rheumatoid arthritis
Anaphylaxis	Ischaemia-reperfusion injury	Sarcoidosis
Ankylosing spondylitis	Multiple sclerosis	Systemic lupus erythematosus
Asthma	Osteoarthritis	Type 1 diabetes mellitus
Atherosclerosis	Pemphigus	Ulcerative colitis
Atopic dermatitis	Periodic fever syndrome	Vasculitides
Chronic pulmonary disease	Psoriasis	Xenograft rejection
Crohn's disease	Gout	
Diseases of infectious origins in which inflammation may contribute as much to the pathology as does microbial toxicity		
Bacterial dysentery	Influenza virus pneumonia	Post-streptococcal
Chagas disease	Leprosy	glomerulonephritis
Cystic fibrosis pneumonitis	Neisserial or pneumococcal	Sepsis syndrome
Filariasis	meningitis	Severe acute malaria
Helicobacter pylori gastritis	Hepatitis C	
Diseases of diverse origin in which post-inflammatory lesions are a principal cause of disease		
Bleomycin-induced pulmonary fibrosis	Hepatic cirrhosis	Schistosomiasis
Allograft rejection	Idiopathic pulmonary fibrosis	Radiation-induced pulmonary fibrosis

Table1.2 - Disorders where underlying inflammatory processes contribute to the pathology. Adapted from[1].

This thesis will focus on T cell-mediated neuroinflammation and the mechanisms that operate in this context to dampen its pathologic outcome. As such, the remaining portion of this chapter will focus on the description of our current understanding of the immune mechanisms underlying the establishment and progression of CNS injury in two pathologies with distinct aetiologies, i.e. multiple sclerosis (MS) and cerebral malaria (CM).

3.1. Multiple sclerosis (MS)

In 1868, Jean Martin Charcot identified MS as a distinct new neurological disease, which he called at the time *sclerose en plaque disseminées*. Charcot not only gave MS its nosological status but also made accurate clinical-pathological correlations, speculated about its pathophysiology and emphasized its frequency[149]. MS is a major chronic inflammatory disease of the CNS with a prevalence estimate of two and a half million individuals affected worldwide[2]. MS has a significant impact on the quality of life for most patients over many years since survival expectancy for MS patients is estimated to be in average 30 years. The disease is believed to arise as a consequence of an autoimmune-mediated attack directed against major constituents of the myelin sheath. Typical clinical symptoms include paralysis, lack of motor co-ordination, sensory disturbances and impaired cognitive functions[150]. Due to its debilitating and chronic nature, MS constitutes a heavy burden not only in the direct costs associated with health care but also in society as a whole with loss of productivity, economic impact on the lives of caregivers and families and wider social welfare costs.

3.1.1. Pathology of MS

MS selectively affects the myelin sheaths and the myelin-forming oligodendrocytes (ODCs) resulting in demyelination and concomitant destruction and degeneration of underlying axons. Demyelination is a consequence of local CNS inflammation, a pathologic hallmark of this disease[151]. Active lesions in MS patients evolve typically around small and medium sized blood vessels with the accumulation of inflammatory infiltrates in the perivascular space. Blood-brain barrier leakage is evident with concomitant extravasation of plasma proteins and cells into the CNS parenchyma[152] as well as haemorrhage in a minority of afflicted patients[153, 154]. Perivascular

infiltrates are composed mainly of subpopulations of lymphocytes consisting of $CD4^+$, $CD8^+$ and γδ T cells, with occasional B cells and plasma cells as well as monocytes/Mø[151]. CNS lesions in MS patients are actively surrounded by activated microglia and Mø expressing high levels of MHC class II[151]. Moreover, infiltrating Mø exhibit a "foamy" phenotype, i.e. myelin-laden, due to the phagocytosis of myelin degradation products. B cells are present to some extent and may produce autoantibodies locally (*see Chapter 1, section 3.1.8.2.*). Actively demyelinating MS lesions can be grouped into four categories on the basis of myelin loss, location and extension of the "plaque", the type of ODC injury and the presence or not of autoantibodies[155]. In pattern I, demyelination is associated primarily with the presence of activated monocytes/Mø in the lesion. Pattern II lesions exhibit autoantibody and complement complexes deposition at the sites of active demyelination together with activated Mø and microglia. Patterns III and IV are highly suggestive of a primary ODC dystrophy. In pattern III, the degeneration of distal ODC processes due to selective loss of myelin-associated proteins, especially myelin-associated glycoprotein (MAG), results in ODC apoptosis and demyelination. Pattern IV lesions, where demyelination is associated with primary ODC degeneration in areas adjacent to active myelin destruction[155], are infrequent in MS patients and appear to be restricted to primary progressive cases. Such heterogeneity in MS lesions suggests that distinct pathologic mechanisms operate in the disease, reflected in the different possible patterns of disease progression (Figure 1.8). Nonetheless, all four lesion patterns are dependent on T cell- and Mø-driven inflammation[155]. Degeneration of axons, a major factor for permanent disability afflicting MS patients, is thought to take place primarily during active demyelination[156]. Release of inflammatory mediators by Mø (*see Chapter 1, section 3.1.8.1.*) and/or cytotoxic activity of CD8+ T cells (*see Chapter 1, section*

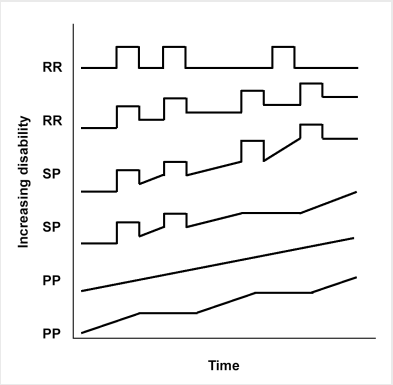

Figure 1.8 - **Clinical courses in MS patients**. In around 80% of individuals suffering from MS, the disease develops initially in a relapsing-remitting (RR) form where episodes of acute exacerbation alternate with periods of full or partial recovery. Disease progression is stable in-between worsening periods. Typically, RR patients evolve over the years into a secondary progressive (SP) clinical course where there is stable and gradual progression of symptoms with or without acute worsening episodes. Other form of MS, i.e. primary progressive (PP), is not associated with distinct periods of acute relapse but rather with a gradual progression of symptoms from the onset of the disease[2]. This latter form is usually correlated with a poorer prognosis.

3.1.8.3.) have been suggested to contribute to axonal degeneration as well. In contrast with the active lesions, in chronic inactive lesions few T cells and plasma cells persist in the perivascular space[157] and astrocytes are actively engaged to substitute the myelin sheath of demyelinated axons resulting in a dense glial scar[158]. Despite the lack of inflammation, axonal degeneration can also occur in inactive lesions[156], possibly explaining the cumulative disability in progressive phases of the disease.

3.1.2. Animal models of MS

It took almost 80 years after the first descriptions of MS to start unravelling the mechanisms of this disease with the establishment of experimental autoimmune encephalomyelitis (EAE), an animal model of antigen-induced autoimmune neuroinflammation. The significance of EAE as an animal model for MS comes from the observation that both share similar clinical and histological features including the course of disease and the occurrence of perivascular leucocytic infiltrates and demyelination within the CNS[159]. The discovery of EAE stems from attempts to explain the occurrence of acute disseminated encephalomyelitis (acute EAE) following Pasteur's antirabic vaccine administration. Since acute EAE was not a direct consequence of rabies virus exposure, Remlinger postulated in 1905 that CNS tissue used in the preparation of the original rabies vaccine propitiated these monophasic paralytic episodes. Remlinger's proposition was later substantiated by Rivers and colleagues, who showed that immunization of primates with brain homogenates produced a pathological condition similar to the postvaccinal paralytic episodes[160, 161]. The observation that emulsification of CNS tissue with adjuvants favoured EAE induction in primates[162] enabled the induction of EAE in mice[163]. Such adjuvants are now known to be DAMPs that trigger the activation of DCs via PRR signalling and consequently that of the adaptive counterpart of the immune system. The establishment of the murine model of EAE laid the grounds for understanding the autoimmune nature of MS. The seminal observations that thymectomized or irradiated mice are resistant to EAE induction, along with the observation that EAE can be induced directly in non-immunized mice by adoptive transfer of previously activated CD4+ T cells as well as the realisation that $CD4^+$ T cell clones with specificity for myelin antigens were capable of mediating EAE provided

the dominant view of self-reactive $CD4^+$ T cells as determinant cellular mediators in the pathogenesis of MS[150].

After earlier attempts to induce active EAE using total brain homogenates, disease induction with purified components of the myelin sheath led to the recognition of myelin basic protein (MBP) and PLP as major encephalitogenic antigens involved in the pathogenesis of this disease. Later, myelin oligodendrocyte glicoprotein (MOG) was added to this list. Topologically, MOG is located in the outermost lamellae of the myelin sheath, which makes it the only identified neuroantigen accessible for both T cell- and autoantibody-mediated damage. The combination of self-reactive T cells and autoantibodies in the pathologic mechanisms operating in murine MOG-induced EAE led to the proposition that this particular system closely reproduces the complex range of clinical and pathological manifestations associated with MS[164]. This prompted us to use MOG-induced EAE as an experimental model to assess the role of HO-1 in the regulation of autoimmune neuroinflammation (*see Chapter 2*)

Experimentally, active demyelination requires the administration of the self-antigen combined with strong immune adjuvants such as complete Freund's adjuvant (CFA). This contains heat-inactivated *Mycobacterium tuberculosis*, responsible for the triggering of PRR signalling in a way as to enhance peripheral inflammation by stimulating the innate immune system and foster a T_H1 response via the induction of IL-12 production by APC[165]. Pertussis toxin, often used in combination with CFA, has been proposed to produce blood brain barrier (BBB) disruption, a critical event for T cell extravasation into the CNS. Furthermore, pertussis toxin administration was shown to strengthen lymphocyte rolling and adhesion due to increased expression of P-selectin in BBB endothelium contributing for increased BBB permeability[166]. Other adjuvant effects of Pertussis toxin include the breakdown of T cell tolerance[167] as well as the enhancement of IFN-γ production by activated T cells[168].

Despite invaluable insight into specific mechanisms associated with MS pathology, EAE is probably an oversimplified version of MS. EAE models are heterogeneous, which make them inadequate to offer an integrated and complete view of the whole spectrum of MS pathology. The intrinsic inducible character of EAE models precludes addressing certain aspects underlying the pathogenesis of MS, such as disease aetiology. Furthermore, clinical progression of EAE is

dependent on the genetic background of the mouse strain and the self-antigen used and does not always reflect that of MS. The same is true for the topology and extension of the demyelinating lesions. While MS lesions are primarily located in periventricular areas, brain stem, optic nerve and upper cervical spinal cord, actively induced demyelination associated with EAE occurs primarily in the lumbar regions of the spinal cord. In addition, EAE is mainly driven by a T_H response and might underestimate the role of $CD8^+$ T cells, B cells and autoantibodies in human MS. Finally, although successful in developing therapeutic strategies to control disease progression, particularly by targeting the inflammatory components of MS, EAE has limitations in evaluating important factors associated with these same strategies. Given the chronic nature of MS, the short-term nature of EAE makes this model inappropriate to access the potential toxic effects of new clinical approaches. Moreover, the risk of opportunistic infectious as a result of chronic immunomodulatory treatments in MS patients is also not easily predicted in EAE models.

3.1.3. Aetiology of MS

The aetiology of MS is highly complex. The current view is that MS susceptibility in a given individual results from the combinatorial effect of both genetic and environmental risk factors. The geographical distribution of MS incidence, best described by increasing prevalence with latitude (both north and south of the equator) (Figure 1.9), suggests that genetic factors contribute to disease susceptibility[169]. Family and twin studies have shown that first-, second- and third-degree relatives of MS-afflicted individuals have a higher probability to develop the disease when compared to the general population and that the associated risk varies with the level of relatedness. For example, the concordance rate of 30% in MS prevalence among monozygotic twins represents a 10-fold increase over dizygotic twins or first-degree relatives[170, 171]. A very representative effort has been done to identify genes that confer susceptibility to MS. Wide genome screens unveiled the

Figure 1.9 - **Geographic distribution of MS incidence**.

strongest association, thought to contribute with 10 to 40% of the genetic risk of developing MS, for one or more genes in the chromosomal area of the human leukocyte antigen (HLA)[172]. Other loci have also been identified in several studies, including TCRβ chain, CTLA-4, ICAM1, chemokine (C-C motif) receptor 5 (CCR5), IL-10, IL-4 receptor α, and IL-2 receptor but their association with MS susceptibility remains controversial[173].

A proposed causative mechanism for the latitude-related distribution of MS is the decrease in sunlight exposure at higher latitudes since UV radiation influences the rate of biosynthesis of vitamin D, considered a natural inhibitor of MS[174]. This view is supported by studies in mice where vitamin D was shown to impair EAE progression [175]. Again, genetic determinants seem to influence the protective role of vitamin D in MS given the association between disease penetrance and a human polymorphism in the vitamin D receptor gene[176].

The gender bias in MS prevalence (women are affected twice as frequently as men) also suggests that other risk factors relate to hormonal differences. This view is supported by i) the observation that MS-afflicted women experience lower relapse rates after pregnancy and worsening of MS during pre-menstrual and menstruation periods ii) the therapeutic effects of the pregnancy-related hormones, e.g. estriol and iii) different gender susceptibility in mice related to the protective effect of testosterone[177]. The opposing effects of sex hormones on the secretion of pro-inflammatory cytokines may explain their influence in MS susceptibility[150]. In addition, experimental evidence suggests that gender bias in disease susceptibility can be related with differences in regulatory T cell activity[178] (*see Chapter 1, section 3.1.9.*).

Notwithstanding, population genetics itself cannot explain MS distribution as emphasized by the high discordance rate of disease penetrance among identical twins[170, 171]. Corroborating evidence for the impact of environmental factors comes from the observation that migration after the age of 15 years from a location of high MS incidence to a location of low incidence carries with it the risk associated with the origin location, while the risk of developing MS after migration before the age of 15 years reflects that of the destination place[169]. An aspect that impacts on MS distribution seems to be related to the economic level of the country. Countries with high standard of health care and sanitation exhibit higher MS prevalence when compared to the so-called undeveloped countries. This led to the proposition of the “Hygiene Hypothesis”, according to which

the propensity to autoimmune diseases such as MS could arise from a reduction in exposure to infectious agents during infancy, allegedly producing an imbalance between T_H1 and T_H2 responses and/or a defect in regulatory T cell populations (*see Chapter 1, section 3.1.9.*).

While the absence of infections is proposed to favour autoimmunity, the occurrence of infections can, in certain conditions, propitiate autoimmune diseases, highlighting the complex relationship between infection and autoimmunity[179]. Infectious agents have been proposed as environmental triggers for a plethora of autoimmune diseases[180]. In accordance, the viral aetiology of human demyelinating diseases such as progressive multifocal leukoencephalopathy, post-infectious encephalitis and subacute sclerosing panencephalitis supports the concept of viruses as potential triggers of MS. Highly seroprevalent members of the herpesvirus family with tropism for the CNS can produce latent and persistent infections. The observation that DNA and viral proteins of the Human herpesvirus 6 have been detected in the cerebrospinal fluid (CSF) and brain of MS patients along with higher lymphoproliferative responses of these patients against viral antigens supports a role for this viral agent in MS[181]. Reactivation of latent Epstein-Barr virus (EBV) infections correlates with MS relapses and antibodies isolated from the CSF of MS patients can cross-react with EBV proteins[182]. The mechanisms by which infectious agents can break down self-tolerance and foster autoimmunity are addressed in the next section.

3.1.4. Inflammation and the breakdown of self-tolerance in MS

Self-tolerance (*see Chapter 1, section 1.2.1.*) is recognized as a major protection against autoimmunity, as varying degrees of self-tolerance breakdown are thought to operate in the establishment of autoimmune diseases. Such immunoregulatory mechanisms aim at controlling adaptive immune responses involving cells with reactivity to self-antigens present in the peripheral repertoire of healthy individuals.

Molecular mimicry, a term reflecting structural homologies between self-antigens and those of microbial organisms, has been proposed as an explanation for how infectious agents would promote the breakdown of self-tolerance and thus favour the emergence of MS. This notion is supported by the highly cross-reactive nature of antigen recognition by T cells[56]. Encephalitogenic T cell clones isolated from MS-afflicted individuals can be re-activated when exposed to viral and

bacterial peptides structurally related with CNS immunodominant self-antigens[183]. Furthermore, analysis of the crystal structure of a TCR from a MS patient revealed that it can recognize both MBP and EBV-derived antigens[184]. In animal models of EAE, immunization with identified cross-reactive peptides results in expansion of self-reactive T cells and CNS infiltration although such immunization protocols failed to produce clinical signs of disease[185]. On the other hand, infection of mice with Theiler's murine encephalomyelitis virus (TMEV) triggers, in a chronic phase, a CNS autoimmune syndrome with similarities to MS in humans[186], providing a functional link between infection and inflammation in the establishment of demyelinating diseases.

Bystander activation of self-reactive T cells and "epitope spreading" have also been proposed as a mean to overcome tolerance and promote MS. Inflammatory reactions associated with infections can potentially result in modulation of innate immunity in a way as to activate self-reactive T cells. In a transgenic mouse model of EAE, APC activation via TLR9 is sufficient to break self-tolerance, resulting in the development of autoimmunity[167]. TLR ligands such as bacterial LPS, CpG or peptidoglycan have been shown to promote activation of self-reactive T cells and EAE establishment[165, 187, 188]. Moreover, mice deficient in the TLR signalling adapter molecule MyD88 are resistant to EAE induction[189]. In this way, it is conceivable that the presence of DAMPs might trigger the activation of otherwise immature DCs presenting self-antigens. This would amplify the immunogenicity of self-antigens, now presented in the context of a fully activated DC, and result in bystander activation of self-reactive T cells. Possibly sinergizing with bystander activation, tissue destruction as a consequence of CNS inflammation in response to local infection can result in *de novo* activation of self-reactive T cells by increasing the diversity of self-antigens available for presentation by activated APCs, i.e. epitope spreading[186, 190].

3.1.5. Lymphocyte entry into the CNS

Irrespectively of the causative agent and/or the antigen source, the establishment of autoimmune demyelination is thought to depend on the peripheral activation of self-reactive $CD4^+$ T cells and their extravasation across the BBB. Demyelinating lesions in MS patients often occur simultaneously in the brain and spinal cord, supporting the notion that self-reactive T cell activation occurs in the periphery rather than within the CNS. This is in accordance with the long lasting

conception that the CNS is an immunologically privileged site. Local anatomical constraints such as the lack of lymphatic drainage and the existence of the BBB are thought to preclude immune reactions within the CNS. The BBB is composed of ECs displaying tight junctions, a critical structure in maintaining the barrier function of the BBB as they act as physical blockade to paracellular movement of immune system cells. Furthermore, the relatively low pinocytic activity, i.e. the unspecific endocytosis of small molecules, and the absence of "fenestrae" in these highly specialized ECs restricts permeability and limits the transcellular movement of plasma proteins. Active transport mechanisms provide the necessary nutrients to meet the demanding metabolic needs of the CNS. Adjoining ECs are perivascular microglia, Mø and CNS-associated DCs surrounded by astrocyte foot processes.

Despite the physical blockage provided by the BBB, immune surveillance does occur in the CNS under homeostatic conditions, as revealed by the presence of leukocytes within the CSF of healthy individuals (Figure 1.10). Lymphocytes with a memory/primed phenotype constitute the major subset present (around 85% and 75% of total $CD4^+$ and $CD8^+$ T cells present in the CSF, respectively), while neutrophils and monocytes are rare (less than 5%)[191]. The different composition of leucocytic subsets present in CSF when compared to peripheral blood suggests that entry into the CNS occurs via a regulated process. Adoptive transfer experiments revealed that entry into the CNS is dependent on the lymphocyte activation state, i.e. activated T cell clones can cross the healthy BBB while naïve T cells do not[192]. The selective entry of activated T cells led to the proposition of a two-step model of lymphocyte extravasation and establishment of neuroinflammation. This model suggests that, under physiologic conditions, entry of activated T cells into the CNS will foster a second large-scale extravasation of lymphocytes and other inflammatory cells that requires the activation of ECs in the BBB. In the absence of inflammation, trafficking of T cells across the BBB depends on the expression of P-selectin in discrete areas of the CNS endothelium, which binds to the P-selectin glycoprotein ligand (PSGL) 1 expressed in memory $CD4^+$ T cells[193]. After the early P-selectin-dependent trafficking into the CNS, T cells that recognize their cognate antigen in the context of a MHC class II molecule acquire an effector phenotype with concomitant production of pro-inflammatory cytokines. Once inflammation is established, subsequent recruitment of antigen-specific T cell clones depends on the interaction of

VCAM-1 and ICAM-1 expressed by activated ECs in the BBB[194, 195] with the $\alpha_4\beta_1$ integrin very late antigen (VLA)-4 and the $\alpha_L\beta_2$ integrin lymphocyte function-associated antigen (LFA)-1 expressed on the surface of activated T_H cells[196]. *In vitro* studies suggest that VLA-4/VCAM-1 interactions mediate firm adhesion of activated T cells to activated ECs[197] while T cell migration across the endothelium is dependent on the interaction of LFA-1 with ICAM-1 and/or ICAM-2[198]. Concomitantly, the ongoing pro-inflammatory milieu also leads to the release of chemokines capable of attracting activated cells via chemokine receptors. A specific role for the chemokines interferon-gamma-inducible protein (IP)-10 and regulated upon activation, normally T-expressed, and presumably secreted (RANTES), and their respective receptors, CXCR3 and CCR5, has been suggested based on the analysis of CNS tissue and SCF from MS patients[199].

After trans-endothelium migration, activated T cells accumulate between the basal membranes of the endothelium and that of the glia limitans, the outermost layer of the CNS composed of the end feet of astrocytes (Figure 1.10), where they secrete matrix metalloproteases responsible for collagenous basal membrane degradation. This is a critical step for BBB disruption and extravasation into the CNS parenchyma[200].

3.1.6. T_H cell reactivation in the CNS

Once in the CNS, activated self-reactive T_H cells must recognize their cognate antigen in the context of a MHC class II to acquire a pathogenic effector phenotype. The observation that, upon adoptive transfer of encephalitogenic T cells, development of EAE requires the presence of blood-derived MHC class II^+ cells in the CNS perivascular space highlights the critical role of these cells in the local reactivation of T_H cell clones[201]. Recently, CNS-associated DCs rather than microglia were identified as being responsible for T cell reactivation upon entry into the CNS[202] (Figure 1.10). Under both physiologic and pathologic conditions, CNS-associated DCs are present in the perivascular space surrounding blood vessels and in the meninges[202-204]. Moreover, during progression of active immunization- or TMEV-induced demyelination, efficient *de novo* activation of naïve T cell clones with specificity to other major antigens in the target tissue other than the disease-inducing one, i.e. epitope spreading, was shown to depend on CNS-associated DCs[203].

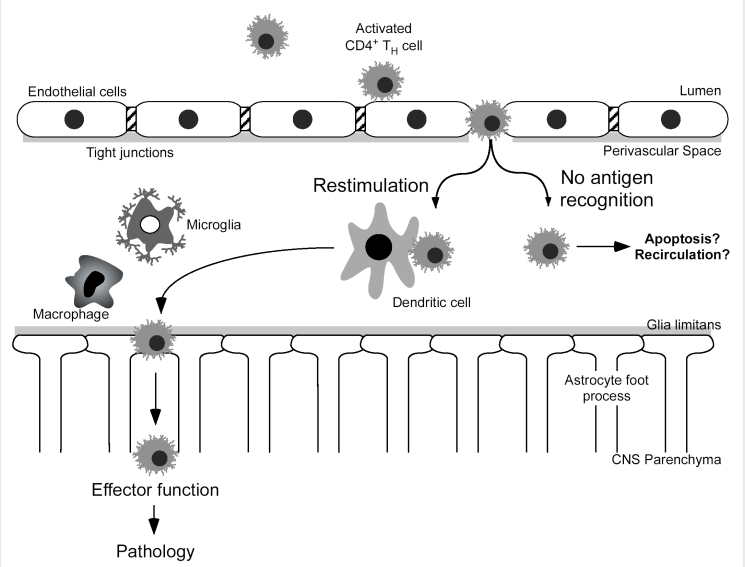

Figure 1.10 - T_H cell reactivation within the CNS. During homeostasis, activated T cells can pass the BBB and circulate in the SCF. However, extravasation into the CNS parenchyma appears to depend on antigen recognition and restimulation by perivascular space associated DCs. T_H cell reactivation within the perivascular space enables autoreactive T_H cells to pass the glia limitans composed of astrocyte foot processes and basal membrane and to enter the brain parenchyma, where they can interact with microglial cells, initiating the inflammatory reaction leading to tissue damage. In the absence of DCs presenting their cognate antigen, self-reactive T_H cells do not pass across the glia limitans and possibly undergo apoptosis or recirculation.

3.1.7. Effector T_H cell function in MS

Until recently the prevalent view was that T_H1 effector T cells mediated tissue damage associated with the development of EAE and presumably that of MS. However, T_H17 cells (Figure 1.4) have emerged as major players in the pathogenesis of autoimmune neuroinflammation. Full effector function and survival of T_H17 cells depends on IL-23, a cytokine that shares a common subunit, i.e. p40, with IL-12. When linked to p35 or p19, p40 forms IL-12 or IL-23, respectively. T_H17 effector cells express IL-17, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF)[205]. The involvement of T_H17 in MS and EAE is underlined by the increased levels of IL-17 in active lesions and CSF of MS patients[206] as well as by the observation that mice deficient in IL-23 ($p19^{-/-}$) are refractory to EAE induction[207]. In accordance, the clinical course of EAE is severely attenuated in IL-17-deficient mice while antibody-mediated IL-17 neutralization resulted in delayed onset or impaired disease progression[208, 209]. IL-17 induces the expression of adhesion molecules in ECs as

well as pro-inflammatory cytokine and chemokine production[205]. Such pro-inflammatory actions are likely to mediate the pathogenic role of T_H17 cells in the context of neuroinflammation by fostering the recruitment and activation of inflammatory cells infiltrating the CNS and promoting tissue damage. In addition, T_H17-driven production of GM-CSF might promote the expansion of Mø infiltrating the CNS during neuroinflammation and promote *de novo* T cell activation within the CNS since this haematopoietic cytokine can stimulate microglia differentiation into DCs[210].

The recognition that the IL-23–IL-17 cytokine pathway has a crucial role in the pathogenesis of EAE has shifted the paradigm of autoimmune neuroinflammation from the IL-12-IFN-γ axis. This is further corroborated by the following set of observations: i) IL-12 (p35) and STAT1-deficient mice are susceptible to EAE induction[211, 212]; ii) genetic disruption of IFN-γ or IFN-γR[213, 214] as well as antibody-mediated blockade of IFN-γ[215] exacerbates rather than attenuates EAE severity and; iii) administration of a recombinant form of this cytokine ameliorates disease[216]. These observations are in sharp contrast with the potential inflammatory role of IFN-γ in human neuroinflammation. Administration of recombinant IFN-γ exacerbates MS while treatment with anti-IFN-γ antibodies improved disease outcome[217]. As such, the apparent independency of autoimmune neuroinflammation concerning IL-12-IFN-γ responses has to be taken with caution. Corroborating evidence for the involvement of T_H1 cells in neuroinflammation is provided by the observation that T_H1 clones can induce EAE upon adoptive transfer and that genetic disruption of T-box expressed in T cells (T-bet), a transcription factor that controls the acquisition of T_H1 phenotype, confers protection against the disease[212]. However, T-bet can also influence T_H17 cell function, given its role in the transcriptional regulation of the IL-23R[218]. As such, resistance to EAE induction in T-bet-deficient mice probably results not only from limiting the development and effector functions of autoreactive T_H1 cells but also from the suppression of T_H17 lymphocytes in the CNS. Analysis for the requirement of IL-12 and IL-23 during the progression of EAE revealed that in mice deficient in the p40 subunit, thus lacking both IL-12 and IL-23, standard EAE progression and severity is only restored by peripheral administration of IL-12 during disease induction followed by IL-23 delivery directly in the CNS[207]. This suggests that the time and tissue localization of IL-12 versus IL-23 responses ultimately dictates the establishment and progression of autoimmune neuroinflammation. Thus, it seems that IFN-γ-producing T cells participate in the onset of

neuroinflammation while local maintenance and/or generation of T_H17 cells is directly responsible for target tissue damage.

3.1.8. Mechanisms of demyelination and axonal damage

Activated encephalitogenic T_H cells initiate autoimmune neuroinflammation by fostering the accumulation of Mø and other inflammatory cells in the brain parenchyma (Figure 1.11). During the active phase of disease, the inflammatory reaction results in tissue damage via the concerted deleterious action of Mø, microglia, T cells, B cells and antibodies. Apart from the direct attack to the myelin sheath of axons, the existence of apoptotic ODCs in demyelinating lesions supports the notion that ODC death contributes to the pathology. Transgenic mice expressing an inducible form of the anti-apoptotic baculovirus-derived caspase-inhibitory protein p35 in mature ODCs exhibit decreased EAE incidence and severity when compared to controls[219], suggesting that ODC apoptosis plays a critical role in the development of EAE. Furthermore, prevention of ODC apoptosis reduces the number of infiltrating inflammatory cells, suggesting that decreasing the availability of self-antigens generated via ODC death dampens inflammation and limits disease progression. The combinatorial effects of axonal demyelination and ODC loss ultimately result in axon malfunction and degeneration responsible for the clinical manifestations associated with MS.

3.1.8.1. Innate cells

Infiltrating Mø and CNS-resident microglia are likely to be the main effector cells responsible for the clinical manifestations of EAE as well as MS (Figure 1.10). This is suggested by the observation that selective depletion of peripheral Mø prevents the onset of EAE[220] whereas blockade of peripheral Mø entry into the CNS reduces disease severity[221]. Moreover, specific inhibition of microglia activation in genetically engineered mice ameliorates clinical progression of EAE while not influencing encephalitogenic T_H1 cell responses[222].

The mechanisms by which Mø and microglial cells promote the pathogenesis of EAE and MS are probably multifactorial. Mø and microglia can sustain neuroinflammation by releasing T cell-activating cytokines, such as IL-12[223] and IL-23[224]. In addition, activated microglia up-regulate the expression of MHC class II[225] and co-stimulatory molecules[203] while phagocytosing myelin[226],

suggesting that these can sustain antigen presentation to encephalitogenic T_H cells. Microglia and Mø are also active participants in myelin breakdown and ODC death via generation of TNF-α and LT-α as well as ROS/RNS and glutamate, all of which are involved in a way or another in loss of axonal integrity (Figure 1.11).

The detrimental role of TNF-R1, as well as that of another member of the TNF receptor superfamily, i.e. Fas, in EAE and MS pathology has been shown to involve the induction of ODC apoptosis. Genetic deletion of TNF-R1 in all tissues and Fas specifically in ODCs confers protection from demyelination and clinical manifestations of the disease[227]. This is in keeping with the observation that exposure of ODCs to cytotoxic cytokines *in vitro* results in death by apoptosis, mediated by caspase 11 activation[228]. While infiltrating mononuclear cells with increased TNF-α production are found in CSF of MS patients and elevated levels of TNF-α and LTα correlate with acute phases of the disease[229, 230], contrarily to EAE[231], antibody-mediated inactivation of TNF-α in MS patients failed to impact or even worsened disease severity in some cases[232]. This suggests that TNF-α might play a role in the regulation of the inflammatory response during ongoing demyelination. In addition, TNF-α has been shown to promote proliferation of ODCs and remyelination[233] suggesting that it might also impact on disease recovery. As for TNF-α, FasL expression is increased in activated microglia and infiltrating T lymphocytes within MS lesions, whereas ODCs in these same lesions display elevated levels of Fas expression[234]. As such, crosslinking of Fas in ODCs by its cognate ligand present in activated microglia can result in ODC apoptosis.

Glutamate is a cytotoxic mediator in the context of neuroinflammation by propitiating oxidative stress. ODCs are highly sensitive to glutamate-mediated excitotoxicity[235], a process by which cells are damaged and eventually die by prolonged stimulation of glutamate receptors, via a mechanisms linked to intracellular Ca^{2+} overload, mitochondrial dysfunction and ultimately to increased ROS production. Similar cytotoxic mechanisms operate in neurons, possibly contributing to the establishment of neurological deficits associated with MS and EAE. Microglia and Mø localized in areas surrounding demyelinated axons express the enzyme responsible for glutamate synthesis, i.e. glutaminase[236] and can produce large amounts of this neurotransmitter[237]. Moreover, increased levels of this excitatory neurotransmitter are detected in the CSF of MS patients and

glutamate receptor antagonists are protective against EAE[238] thus suggesting that this pathway may operate in the pathogenesis of MS.

Other sources of oxidative stress leading to ODC and/or neuron toxicity are associated with the production of high levels of ROS and RNS via the activation and/or induction of the NADPH and iNOS systems in Mø and microglia cells. Both TNF-α and IFN-γ can induce the expression of iNOS as well as that of NADPH in microglia, astrocytes and Mø. iNOS expression is detected in active

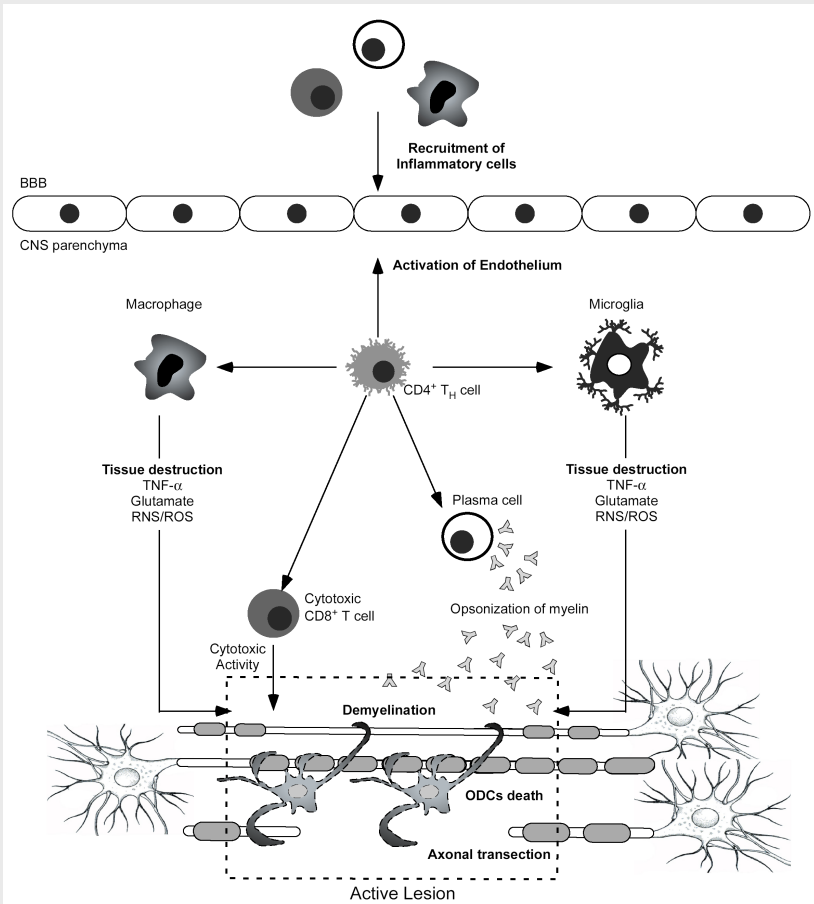

Figure 1.11 - **Pathogenesis of Multiple Sclerosis**. Upon entry and reactivation within the CNS, autoreactive T_H cells secrete pro-inflammatory cytokines (IFN-γ and/or IL-17) required for the establishment and maintenance of a local pro-inflammatory environment. This result in the activation of BBB ECs with the concomitant recruitment of inflammatory cells, activation of M and microglia and the differentiation of B cells into autoantibody-secreting plasma cells. Activated Mø and microglia produce cytotoxic cytokines (TNF-α) and molecular mediators (ROS/RNS and glutamate) that collaborate with anti-myelin antibodies and cytotoxic CD8+ T cells to cause demyelination, axonal damage and ODC death, ultimately responsible for the neurological deficits associate with the disease

MS lesions, mainly in activated microglia and Mø. Increased levels of NO are present in the serum and CSF of MS patients as well as during EAE and correlate with disease severity. Evidence for lipid peroxidation in plasma and CSF of MS patients supports the deleterious role of these oxidizing mediators in promoting tissue damage during disease[239]. Phagocytosis of myelin by Mø is dependent on the generation of ROS[240] and NO has been shown to mediate microglia-dependent killing of ODCs[241]. In addition from its cytotoxic effects, NO has other deleterious effects that can contribute to the pathogenesis of MS, such as to promote the secretion of pro-inflammatory cytokines and other inflammatory mediators[242], deregulate BBB function[243] and promote the activity of MMPs involved in several pathogenic mechanisms in demyelinating disease[244].

3.1.8.2. B cells

The role of B cells in human autoimmune demyelination is poorly understood, especially given their relative irrelevance in disease progression in most EAE models (*see Chapter 1, section 3.1.2.*). Potential mechanisms via which B cells and autoantibodies contribute to the pathogenesis of autoimmune demyelination have been proposed to involve the opsonization of myelin (Figure 1.11), thus increasing its phagocytosis by infiltrating Mø[245] and ultimately resulting in augmented TNF-α and NO production[245]. Other mechanisms might relate to activation of complement-mediated lysis of target cells[246]. Nevertheless, recent evidence from mice genetically deficient in central components of the complement cascade dismiss the importance of the membrane attack complex (MAC) in the pathogenesis of EAE[247]. In MS patients, B cells, plasma cells and autoantibodies are present in active demyelinating lesions[155]. Serological studies reveal increased Ig content in the CSF of 90% of MS patients. The high frequency of oligoclonal antibodies in the CSF of MS patients points to antigen-specific selection and clonal expansion of B cells during neuroinflammation[248]. Nevertheless, antibody specificity has been difficult to assess in the context of MS. Autoantibodies with specificity to neuroantigens do not correspond to the main oligoclonal serotypes detected in CSF from MS patients, possibly due to BBB leakage, which facilitates the entry of peripherally produced antibodies into the CNS. Yet, antibodies with specificities to major neuroantigens, i.e. MOG, are detectable in the CSF of MS patients[249] and associate with

disintegrating myelin surrounding axons in active lesions in MS patients[250] pointing to a role in mediating target tissue damage.

3.1.8.3. CD8+ T cells

During the progression of neuroinflammation, activation of CD8+ T cells can be triggered upon TCR engagement by DCs presenting neuroantigens in the context of appropriate MHC class I molecules. Activated self-reactive T_H cells can contribute to $CD8^+$ T cell activation either by licensing DCs, i.e. promoting the expression of co-stimulatory molecules and other activating signals required for $CD8^+$ T cell activation in a CD40/CD40 ligand dependent way, or by providing growth factors. Once activated, myelin-specific $CD8^+$ T cells are capable of directly mediating tissue destruction in a MHC class I-restricted way by lysing cells expressing the appropriate ligand via the production of lytic mediators (perforin and granzyme)- or a FasL-mediated mechanism (Figure 1.11). Although both $CD4^+$ and $CD8^+$ T cells are present in the CNS during neuroinflammation, $CD8^+$ T cells are the predominant lymphocytes detected in MS lesions[251]. Oligoclonal expansion of $CD8^+$ T cells is often found in MS patients whereas clonal expansion of $CD4^+$ T cells is rarely observed[251]. Furthermore, $CD8^+$ T cells from MS patients exhibit increased adhesiveness to inflamed endothelium when compared to T_H cells, suggesting that $CD8^+$ T cells have enhanced capacity to cross the BBB and infiltrate the CNS parenchyma[252]. Upon adoptive transfer, MBP-specific and MOG-specific cytotoxic $CD8^+$ T cells can mediate clinical manifestations associated with autoimmune demyelination[253, 254]. However, adoptive transfer of MOG-specific $CD8^+$ T cells in β_2-microglobulin deficient mice, i.e. with no MHC class I expression, fails to produce clinical signs of EAE[254], suggesting that the pathogenic effects of $CD8^+$ T cells depends on the expression of MHC class I molecules in the target tissue. Evidence for the cytotoxic role of $CD8^+$ T cells during CNS inflammation is provided by *in vitro* observations. Human primary ODCs are targets of MBP-specific $CD8^+$ T cell-mediated lysis in the absence of exogenous antigen, an effect abrogated by antibody-mediated blockade of MHC class I[255]. This observation suggests that ODCs process and present endogenous antigen in such a way as to become susceptible to cytotoxic $CD8^+$ T cell-mediated killing. Similarly, neurons expressing MHC class I following IFN-γ exposure are directly damaged by cytotoxic $CD8^+$ T cells[256].

Despite the generally accepted pathogenic role of CD8+ T cells in CNS inflammation, a protective role of $CD8^+$ T cells has also been proposed. EAE induction in mice lacking $CD8^+$ T cells, either by genetic engineering or antibody-mediated depletion, results in higher incidence of relapse episodes[257, 258]. In addition, suppression of EAE by tolerogenic APCs was shown to act via the induction of regulatory population of $CD8^+$ but not T_H cells[259]. Several studies suggest that such regulatory $CD8^+$ T cells counteract neuroinflammation by limiting the expansion of self-reactive T_H cells via apoptotic mechanisms[260].

3.1.9. Regulatory mechanisms that counter neuroinflammation

The clinical course of MS is, in most cases, associated with transitory episodes of remission (*see Chapter 1, section 3.1.1.*), suggesting that regulatory mechanisms operate to counter disease progression. Clinical recovery involves dampening of neuroinflammation, allowing mechanisms such as remyelination and restoration of neuronal electrical conductivity to proceed. In addition, functional reorganization of neuronal networks can compensate for neurological deficits following demyelination[261].

Cytokines are major players in orchestrating all phases of autoimmune neuroinflammation. While T_H1 and T_H17 cytokines are present in the CNS during the initiation and active phase of EAE and MS (*see Chapter 1, section 3.1.7.*), disease remission is correlated with the presence of cytokines corresponding to a T_H2 phenotype[262]. Cytokines expressed by T_H2 cells, including IL-4, IL-10 and IL-13, have been suggested to have a significant role in suppressing EAE. IL-25, a T_H2 promoting member of the IL-17 family of cytokines affords protection against EAE, i.e. mice lacking IL-25 expression exhibit exacerbated EAE, while exogenous IL-25 administration inhibits EAE development and progression [263]. The protective effect of IL-25 depends on its ability to dampen T_H17 differentiation and function by promoting T_H2 responses and in particular the production of IL-13, a prototypical T_H2 cytokine that acts on DCs to suppress the expression of T_H17 promoting factors, namely IL-23 and IL-6.

Similarly, the role of IL-4 in suppressing neuroinflammation is well documented. Adoptive transfer of neuroantigen-specific T_H2 cells producing IL-4 at the time of immunization inhibits EAE and promote disease remission if transferred after clinical disease is established[264]. In accordance,

adoptive transfer of encephalitogenic T cells over-expressing IL-4 through retroviral infection after active immunization results in delayed onset and reduce disease severity[265]. However, IL-4-deficient mice have a clinical course of disease similar to wild-type controls. In contrast, IL10-deficient mice are highly susceptible to EAE, an effect associated with increased T cell proliferation and T_H1 cytokine production. Moreover, transgenic mice over-expressing IL-10 are resistant to EAE induction[266]. Therefore, it is possible that the regulatory effects afforded by IL-4 are due to its capacity to promote immune deviation towards a T_H2 effector phenotype with IL-10 and IL-13 being critical effector cytokines in the regulation of EAE progression.

The importance of T_H2 cells in dampening neuroinflammation led to the development of several strategies with potential therapeutic benefits. For example, among their pleiotropic effects in the context of neuroinflammation[267], statins promote the induction of a T_H2 response[268]. The T_H2 shift in effector function of encephalitogenic T cells is suggested to contribute, at least in part, to the reduction of clinically established disease and/or prevention of relapse episodes. Another therapy, based on the use of altered peptide ligands (APL), which consist of antigenic peptides with aminoacid substitutions in positions required for TCR binding, has also been shown to affect the T cell effector phenotype balance crucial for disease outcome[269-271].

CD4+ T cell populations with a regulatory phenotype constitute another level of regulation in the control of neuroinflammation. In a model of spontaneous EAE, transgenic mice with $CD4^+$ T cells expressing a TCR specific for the MBP peptide consisting of aminoacids 1 to 11 (T/R^+) remain healthy throughout life. However, these mice develop a lethal form of EAE spontaneously once the TCR repertoire was restricted to the transgenic TCR by crossing with mice deficient for αβ TCR or Rag genes (T/R^-). This suggests that protection from disease depends on the presence of a $CD4^+$ regulatory T cell population expressing endogenous αβ TCR[272, 273]. In fact, T/R^+ mice contain MBP-specific $CD4^+CD25^+T_{regs}$ which are absent in T/R^- mice[274]. Moreover, transfer of peripheral $CD4^+$ T cells from normal mice into T/R^- before clinical onset of EAE precludes disease development[273]. Active immunization models of neuroinflammation have provided further evidence for the role of T_{regs} in controlling the establishment of CNS autoimmunity. Regulatory T cells accumulate within the CNS during EAE and are very efficient in suppressing T_H cell responses *in vitro* as well as in reducing clinical manifestations of EAE when transferred before immunization[275]. In MS patients,

regulatory T cells, albeit exhibiting normal frequencies in peripheral blood and CSF when compared to healthy individuals, display an intrinsic defect in their *in vitro* suppressor activity, an effect associated with reduced levels of Foxp3 expression[276, 277].

In addition from dictating the establishment of CNS inflammation, T_{regs} impact in disease progression upon active immunization. Transfer of peripheral T_{regs} from non-immunized animals diminishes EAE severity following immunization[278] while antibody-mediated depletion or inactivation of T_{regs} results in increased severity during the acute phase of disease and prevents secondary remissions[279]. Recent evidence however, has challenged the notion that, under inflammatory conditions as it occurs in the CNS during disease progression, T_{regs} are capable of modulating local inflammation[280]. By using a transgenic mouse model in which anti-MOG_{35-55} T_{regs} express GFP, these self-antigen specific T_{regs} were shown to expand and infiltrate the CNS after active immunization, but failed to inhibit antigen-specific responses of T_H cells isolated from the CNS. This was shown to be due to an intrinsic resistance of T_H cells. Such effect is associated with the presence in the CNS of IL-6, a pro-inflammatory cytokine that renders T_H cells refractory to T_{reg}-mediated suppression and thus enables immune responses in the presence of regulatory T cell populations[281]. Given the observation that IL-17 induces the expression of IL-6, IL-17 might be responsible for bypassing the regulation afforded by T_{regs}. In keeping with these observations, transfer of MBP specific $CD4^+CD25^+T_{regs}$ cells into T/R^- before EAE onset affords complete protection while their administration in mice with ongoing disease has no clinical benefit. Taken together these observations suggest that while T_{regs} are efficient in regulating responses in the periphery, the local presence of inflammatory cytokines hampers T_{reg}-mediated suppression in the target tissue.

3.2. Cerebral malaria (CM)

According to the World Health Organization estimates published in the World Malaria Report of 2005 (http://rbm.who.int/wmr2005/index.html), approximately 350-500 million individuals are infected with malaria, with a total death rate ranging from 1.3 to 1.5 million annually. The highest incidence of mortality occurs in children less than 5 years old. From the four species of intracellular protozoan parasites of the genus *Plasmodium* capable of infecting humans, *P. falciparum* is the

main responsible for the occurrence of severe disease complications, a syndrome known as severe malaria (SM). The aetiology of SM is highly complex as malaria fatalities are often associated either with systemic, single or multi-organ failure. These include acute respiratory distress, shock, renal failure and pulmonary oedema, with the most prevalent one being CM[282]. This neurological dysfunction is characterized clinically by impaired consciousness, non-specific fever, generalized convulsions and unrousable coma.

The *Plasmodium* life cycle in humans starts with its inoculation into the bloodstream by the bite of an infected female *Anopheles* mosquito. Within half an hour, sporozoites reach the liver and invade hepatocytes, initiating the so-called liver stage of infection[283]. Within hepatocytes, *Plasmodium* parasites undergo several cycles of asexual division, after which thousands of erythrocytic merozoites are released from each liver cell and infect circulating RBCs, a life cycle phase known as blood stage. After going through several stages within the RBC, erythrocytic merozoites are released via RBC cell lysis and immediately invade new ones. A small proportion of the merozoites undergo transformation into gametocytes that can be ingested by a mosquito when it bites an infected individual. If not controlled by immune mechanisms or anti-malarial drugs, blood parasite load, i.e. parasitaemia, and RBC infection/lysis will increase exponentially resulting in severe anaemia and death. The innate and adaptive immune system mechanisms that control parasitaemia are beyond the scope of this thesis and therefore shall not be addressed. Indeed, the majority of malarial infections result in a chronic debilitating disease rather than in the acute manifestations of SAM. However, a proportion of infected individuals develop the rare symptoms of complicated neurological disease, i.e. CM, mediated by malarial erythrocytic stages.

The mechanisms involved in CM establishment were once thought to be strictly correlated with the sequestration of parasite-infected RBC in brain microvasculature leading to vascular obstruction and cerebral hypoxia[284]. However, RBC sequestration does not explain by itself the occurrence of the pathology. Local and systemic inflammation has emerged as a major factor associated with the development of CM[285] (Figure 1.12). A conciliatory hypothesis for the pathogenesis of CM has been proposed where parasite-induced adhesion of erythrocytes to ECs in the brain microvasculature directs the accumulation of immunostimulant parasite products and triggers a local cascade of pro-inflammatory events leading to pathology[286]. Animal models, even if not recapitulating all the features of human CM, have provided valuable contributions in

understanding the mechanisms operating in the pathology. For example, in the *P. berghei* ANKA murine model, the neurological-associated symptoms of infected mice, i.e. ataxia, hemiplegia and coma, are similar to the clinical features of human CM.

During the malarial blood stages of *P. falciparum* infection, parasite-derived soluble products such as the glycosylphosphatidylinositol (GPI) membrane anchor of several glycoproteins and hemozoin (hydrophobic heme polymers resulting from parasite metabolism of host hemoglobin) have been shown to activate innate cells via TLR2/TLR4[287] and TLR9[288] respectively. The role of these PRRs in promoting the pathogenesis of CM is illustrated by the fact that mouse strains susceptible to CM have decreased lethality when TLR2 or TLR9 expression is deleted by homologous recombination[289]. Furthermore, a polymorphism in the human TLR4 gene was shown to be associated with increased CM incidence[290]. The activation of host innate cells via PRR activation results in overproduction of pro-inflammatory cytokines and DC activation, providing the required signals required for T_H cell polarization into an effector phenotype (Figure 1.12). In fact, parasitised RBC can up-regulate the expression of MHC class II and co-stimulatory molecules in DCs and induce the production of IL-12[291], a paramount cytokine in the induction of a T_H1 response. Furthermore, splenic DCs from infected mice are able to activate naïve $CD4^+$ T cells to produce IL-2 and IFN-γ[292].

The role of T lymphocytes in the pathogenesis of CM is suggested by the following set of observations. T_H1-dependent cytokines, i.e. IFN-γ, have a crucial role in the pathogenesis of ECM as both IFN-γ– and IFN-γR-deficient mice are resistant to CM[293]. Depleting antibodies against either T_H or $CD8^+$ T cells provide protection against CM[294]. Likewise, mice deficient in the T cell compartment (severe combined immunodificient (SCID)) do not develop ECM[294]. Analysis of the kinetics for the requirement of distinct T cell populations has revealed that depletion of $CD4^+$ T cells early in infection prevents the development of CM whereas depletion immediately before onset of clinical symptoms does not[295]. This suggests that T_H cells are involved in the induction of CM rather than directly involved in disease progression. The fact that both CD40 and CD40L are required for CM-associated mortality[296] suggests that, following activation and T_H1 skewing, $CD4^+$ T cells provide help to $CD8^+$ T lymphocytes possibly by licencing DCs in a CD40/CD40L-dependent manner. Accumulation of $CD8^+$ T cells in the brain occurs simultaneously with the neurological

signs of the disease and CD8+ T cell depletion 24 hours before the expected clinical signs prevents the development of CM[295], suggesting that CD8+ T cells are responsible for the manifestations associated with ECM. Once primed, cytotoxic effector CD8+ T cells migrate to the brain where they interact with EC lining the brain microvasculature and mediate injury (Figure 1.12). Cytokine-activated EC can up-regulate expression of MHC complexes[297] and internalize/phagocyte infected RBC[298]. In this way, parasite-derived antigens may be presented, in the context of MHC class II and/or class I, at the cell surface of ECs. This suggests that antigen recognition by cytotoxic effector CD8+ lymphocytes through their TCR might induce EC killing via a perforin- and granzyme-dependent pathway[299], resulting in disturbance of BBB function. Impairment of BBB ECs can result in extravasation of plasma proteins and parasite toxins that, under physiologic conditions, would not gain access to the brain parenchyma resulting in neuropathological injury and death.

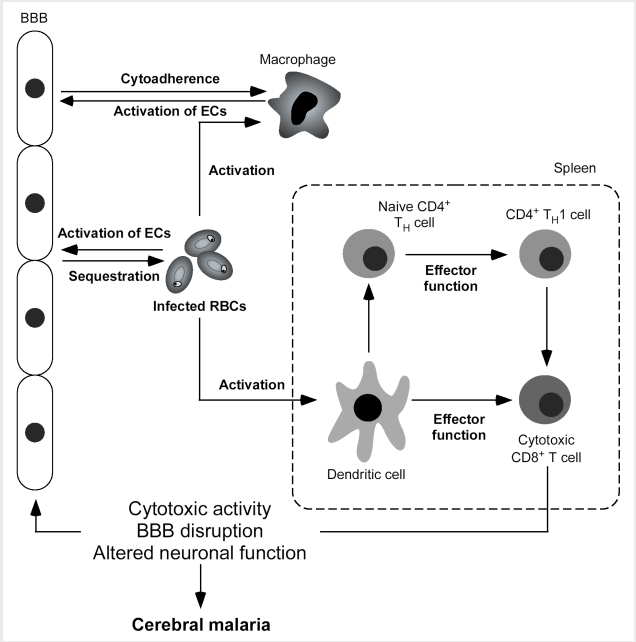

Figure 1.12 – Pathogenesis of Cerebral Malaria. Infected RBCs interact with activated ECs in brain microvasculature. In parallel, systemic activation of innate immune cells via TLR recognition of parasite-derived molecules result in activation of innate system cells. Activated Mø are attracted locally and foster a pro-inflammatory environment. DCs promote an adaptive response by activating CD4+ and CD8+ lymphocytes, an event thought to occur in the spleen. T_H1-polarized CD4+ lymphocytes provide help to CD8+ cells that in turn migrate to the brain. Cytotoxic CD8+ lymphocytes are though to foster BBB disruption via perforin-dependent killing of ECs, and to alter neuronal function by secreting IFN-γ. As a result, CNS metabolism is altered and cerebral function is compromised.

Other adverse effects of activated CD8+ cells that might contribute for the pathogenesis of CM relate to their role as potential sources of INF-γ. This pro-inflammatory cytokine might have an important role in brain metabolic changes during malaria infection. INF-γ acts as a strong inducer of idoleamine 2,3-dioxygenase (IDO)[300], the rate-limiting enzyme in the kynurenine pathway of tryptophan metabolism. This pathway generates the metabolites

kynurenic acid (KA), considered a neuroprotectant, and quinolinic acid (QA), a mediator of excitotoxicity[301]. Increased IDO activity and imbalanced ratio of brain QA to KA is associated with the latter stages of murine CM[302]. Significant changes in the ratio of these metabolites were also associated with a fatal outcome in CM-afflicted patients[303]. IFN-γ also mediates FasL expression on activated microglia[304] and promote astrocyte apoptosis[305]. Given that astrocytes convert kynurenine preferentially into the neuroprotectant KA while microglia and Mø generate the neurotoxic QA[306], loss or interference with the normal functions of astrocytes would impair neuronal function, possibly contributing to the development of CM.

4. Aims and thesis outline

The research presented in this doctoral thesis aimed at a better understanding of the impact of HO-1 expression and/or that of its end products over key cellular mechanisms involved in the establishment and progression of inflammatory diseases. We have done so in two distinct types of inflammatory diseases that target the CNS. We studied the physiologic role of HO-1 expression in the pathogenesis of autoimmune neuroinflammation, i.e. MS, (*see Chapter 2*) and addressed the significance of induction of HO-1 expression and/or exogenous CO administration from a therapeutic point of view. Directly linked to this study, we addressed whether HO-1 expression impact the regulatory capacity of naturally occurring regulatory T cells, T_{regs}, as this cellular subset has been shown to have a crucial role in the regulation of inflammatory diseases including autoimmune neuroinflammation (*see Chapter 3*). Further, we studied the impact of pharmacological induction of HO-1 expression or exogenous CO administration in another type of inflammatory disease affecting the CNS, i.e. ECM due to severe disease complications during malarial infections (*see Chapter 4*).

Chapter 2: Heme Oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation

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1. Abstract

Heme oxygenase-1 (*Hmox-1*/HO-1) dampens inflammatory reactions via the catabolism of heme into carbon monoxide (CO), iron and biliverdin. We report that expression of HO-1 can dictate the pathologic outcome of experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS). Induction of EAE in C57BL/6 *Hmox-1*$^{-/-}$ mice led to enhanced central nervous system (CNS) demyelination, paralysis and mortality, as compared to *Hmox-1*$^{+/+}$ mice. Induction of HO-1, by cobalt protoporphyrin IX (CoPPIX) administration after EAE onset, reversed paralysis in C57BL/6 and SJL/J mice and disease relapse in SJL/J mice. These effects were not observed using zinc protoporphyrin IX, which does not induce HO-1. CoPPIX protection was abrogated in *Hmox-1*$^{-/-}$ C57BL/6 mice, indicating that CoPPIX acts via HO-1 to suppress EAE progression. The protective effect of HO-1 was associated with inhibition of major histocompatibility complex class II expression by antigen presenting cells and inhibition of T_H and CD8 T cell accumulation, proliferation and effector function within the CNS. Exogenous CO mimicked these effects, suggesting that CO contributes to the protective action of HO-1. In conclusion, HO-1 or exposure to its end product CO counters autoimmune neuroinflammation and thus might be used therapeutically to treat MS.

2. Introduction

Multiple sclerosis (MS) is a presumed autoimmune disorder that targets the central nervous system (CNS)[1]. Neuroinflammatory lesions associated with MS progression are triggered upon interaction of activated pathogenic T helper (T_H) cells with antigen presenting cells (APC) within the CNS. This leads to the generation of a proinflammatory response that causes irreversible oligodendrocyte injury, neuron demyelination and eventually axonal loss, the main pathological hallmarks of MS[2-4].

The clinical course of MS is, in most cases, associated with transitory episodes of remission[2], suggesting that regulatory mechanisms must operate to counter neuroinflammation and/or to promote neuron regeneration. Such mechanisms may involve, but are most probably not limited to, the participation of regulatory T cells[5,6]. We hypothesized that expression of "protective genes"[5,7] outside the regulatory T cell compartment might also promote MS remission. One candidate is heme-oxygenase-1 (*Hmox-1*/HO-1), a prototypical cytoprotective and anti-inflammatory stress-responsive gene (*reviewed in*[5,8]) expressed in the CNS, during the course of MS[9] and experimental autoimmune encephalomyelitis (EAE)[10], a well established model of MS[11].

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[12], which is subsequently reduced into bilirubin by biliverdin reductase (*reviewed in*[13]). Induction of HO-1 by metal protoporphyrins has been shown to have salutary effects in a variety of experimental inflammatory conditions (*reviewed in*[8,14]). The observation that in most cases, exposure to CO can mimic the protective effects of HO-1 (*reviewed in*[8,15]) would suggest that HO-1 acts in a protective manner via the generation of CO. It is likely however, that other end products of HO-1 activity such as biliverdin[16] and/or free Fe (by up-regulating heavy chain ferritin expression[17] or cellular Fe efflux pumps[18]) may exert similar effects. Whether HO-1 modulates the pathogenesis of autoimmune neuroinflammation remained to be established since both protective[19] and deleterious[20] effects of chemical HO-1 modulators have been demonstrated in EAE. Using HO-1-deficient (*Hmox-1*$^{-/-}$) mice, we demonstrate unequivocally that expression of HO-1 inhibits inflammation, demyelination and paralysis, preventing mortality associated with the development of EAE. We also provide evidence that induction of HO-1 using protoporphyrins can modulate ongoing autoimmune neuroinflammation, thereby reverting paralysis and leading to disease remission in mice with

previously established EAE. Exogenous CO can mimic this effect, suggesting that this end-product of heme degradation contributes to the protective action of HO-1. Suppression of EAE was associated with inhibition of i) leukocyte accumulation in the CNS, ii) major histocompatibility complex class II (MHC class II) expression by CNS antigen presenting cells (APC) and iii) pathogenic T_H cell proliferation and effector function.

3. Results

3.1. Expression of HO-1 counters the pathogenesis of EAE

Given that HO-1 expression in the CNS is associated with the development of both EAE and MS[10,19,20] and based on the well-established protective effect of HO-1 in a variety of inflammatory conditions (*reviewed in*[5,8]), we hypothesized that HO-1 might modulate the pathogenesis of EAE. When immunized with the myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) in complete Freund´s adjuvant (CFA), HO-1-deficient (*Hmox-1*$^{-/-}$) C57BL/6 mice developed a more severe form of EAE characterized by increased paralysis, as compared to wild type (*Hmox-1*$^{+/+}$) C57BL/6 mice (Figure 2.1A and Table 1). Moreover, EAE lead to 75% mortality in *Hmox-1*$^{-/-}$ mice, as compared to 19% mortality in *Hmox-1*$^{+/+}$ controls (p=0.0024)(Table 2.1). Heterozygous (*Hmox-1*$^{+/-}$) mice behaved in a similar manner to wild type *Hmox-1*$^{+/+}$ mice (*data not shown*).

Table 2.1 HO-1 controls the pathogenesis of EAE

	Genotype	n	Incidence (%)	Onset (day±SEM)	Maximal disease Score±SEM	Mortality (%)
I	*Hmox-1*$^{+/+}$	21	95	16±1.01	2.3±0.35 (day 26)	19.04
II	*Hmox-1*$^{-/-}$	8	100	15.5±0.73	4.5±0.37 (day 40)(1)	75(2)

(1) p= 0.0053 *versus* group I; (2) p=0.002 *versus* group I.

We tested whether induction of HO-1 by metal protoporphyrins would arrest EAE progression in wild type *Hmox-1*$^{+/+}$ C57BL/6 mice. Induction of HO-1 expression/function by administration of cobalt protoporphyrin IX (CoPPIX) after EAE onset (i.e. 37% disease incidence; 0.7±0.1 mean disease score) reversed paralysis and led to complete disease remission in 66.6% of mice with previously established EAE (Figure 2.1B and Table 2.2). This did not occur in control mice treated with vehicle (phosphate buffered saline; PBS) or zinc protoporphyrin IX (ZnPPIX) (Figure 2.1B and Table 2.2).

To address whether similar effects would occur in other mouse strains, EAE was induced in SJL/J mice, by immunization with the proteolipid protein peptide 139-151 ($PLP_{139-151}$) in CFA. Induction of HO-1 by administration of CoPPIX after EAE onset in SJL/J mice (40% disease incidence; 0.8±0.2 mean clinical score) reversed paralysis and lead to complete disease remission

in 75% of mice with previously established EAE (Figure 2.1C and Table 2.2). Moreover, disease relapse was also suppressed in SJL/J mice treated with CoPPIX (Figure 2.1C and Table 2.2). The

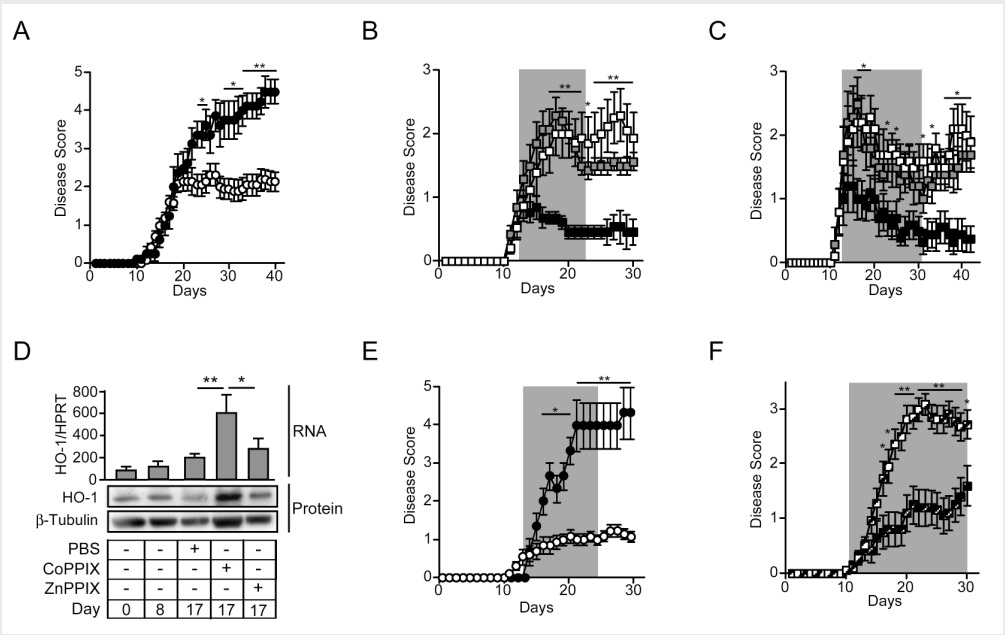

Figure 2.1- HO-1 and CO suppress EAE progression. EAE was induced at day 0 and disease severity scored daily thereafter, as described in Methods. Clinical scores are shown as mean ± standard error of mean (*p<0.05, **p<0.01). (**A**) Progression of EAE in C57BL/6 *Hmox-1*$^{+/+}$ (○)(n=21) versus *Hmox-1*$^{-/-}$ (●)(n=8) mice. (**B** and **C**) EAE was induced in C57BL/6 (**B**) or SJL/J (**C**) mice, randomized two days after disease onset and treated daily with PBS (○)(n=10-15), CoPPIX (■)(n=10-14) or ZnPPIX (■)(n=10-14), as described in Methods. Shaded areas indicate periods of treatment. (**D**) C57BL/6 *Hmox-1*$^{+/+}$ were treated as in (B). HO-1 mRNA and protein expression in the CNS were assessed by qRT-PCR and western blotting, respectively. Expression HO-1 mRNA is shown as mean number of HO-1 per HPRT mRNA molecules ± standard deviation. (**E**) EAE was induced in C57BL/6 *Hmox-1*$^{+/+}$ (○)(n=12) and *Hmox-1*$^{-/-}$ (●)(n=3) mice treated with CoPPIX as in (B). (**F**) EAE was induced in C57BL/6 *Hmox-1*$^{+/+}$ mice, randomized 10-12 post-immunization and exposed to CO (n=20)(450ppm)(□) or not (n=22)(□). Shaded area indicates period of CO exposure.

observation that induction of HO-1 suppressed EAE progression in C57BL/6 as well as in SJL/J mice, excludes the possibility that the protective effect of HO-1 would be specific to a given mouse strain, i.e. C57BL/6 versus SJL/J or to a specific antigen, i.e. MOG_{35-55} *versus* $PLP_{139-151}$, respectively.

That CoPPIX induced HO-1 expression in C57BL/6 mice with established EAE was confirmed at the mRNA and protein level, by qRT-PCR and western blot, respectively (Figure 2.1D). CoPPIX induced a 3-fold increase in HO-1 mRNA expression in the CNS, when compared to immunized PBS-treated controls (p<0.01) (Figure 2.1D). HO-1 mRNA was not significantly induced

upon ZnPPIX administration (Figure 2.1D). Expression of HO-1 protein was confirmed using protein extracts from the CNS of CoPPIX-, ZnPPIX- and vehicle-treated mice (Figure 2.1D).

When used at the same dose and schedule shown to arrest EAE progression in wild type *Hmox-1*$^{+/+}$ C57BL/6 mice (Figure 2.1B), CoPPIX failed to arrest EAE progression in *Hmox*-1$^{-/-}$ C57BL/6 mice (Figure 2.1E). This observation confirms that the protective effect of CoPPIX requires the expression of HO-1. We have previously shown that exogenous CO can mimic the protective effects of HO-1 in several inflammatory conditions[21](*reviewed in*[8]).

Table 2.2 Induction of HO-1 or CO exposure suppresses EAE

	Strain Background	Treatment	n	Maximal disease Score±SEM	Mortality (%)	Remission (%)
I	C57BL/6	PBS	15	2.33±0.44 (day 28)	20	0
II	C57BL/6	CoPPIX	14	0.84±0.21 (day 15)(1)	0	66.6(4)
III	C57BL/6	ZnPPIX	14	2.28±0.22 (day 18)	20^{ns}	0
IV	SJL/J	PBS	10	2.3±0.36 (day 20)	0	11.1
V	SJL/J	CoPPIX	10	1.2±0.45 (day 16)(2)	0	75(5)
VI	SJL/J	ZnPPIX	10	2.5±0.44 (day 17)	0	11.1
VII	C57BL/6	Air	10	3.09±0.26 (day 23)	22.7	0
VIII	C57BL/6	CO	10	1.9±0.69 (day 30)(3)	15^{ns}	0

(1) p= 0.0117 *versus* group I. (2) p= 0.0278 *versus* group IV. (3) p= 0.009 *versus* group VII. (4) p<0.05 *versus* group I and III; (5) p<0.05 *versus* group IV and VI.

To determine whether this was the case in EAE, wild type C57BL/6 mice were exposed to CO via inhalation (450 parts per million; ppm), starting 10 or 12 days post-immunization, when EAE incidence was 17% and the mean clinical score was 0.3±0.1. The effect of CO was compared to that of air, inhaled under similar flow conditions. CO arrested EAE progression and paralysis (Figure 2.1F and Table 2.2). A significant protective effect was also observed when lower levels of CO (250 ppm) were used, but this was somewhat less effective (*data not shown*). We asked whether expression of HO-1 during the course of EAE prevented CNS demyelination, the main

cause of paralysis associated with EAE (*reviewed in* [2-4]). CNS demyelination was more pronounced in *Hmox-1*$^{-/-}$ versus *Hmox-1*$^{+/+}$ C57BL/6 mice, as assessed 60 days after the induction of EAE (Figure 2.2A-F). Only low-grade leukocyte CNS infiltration was observed, most probably due to the

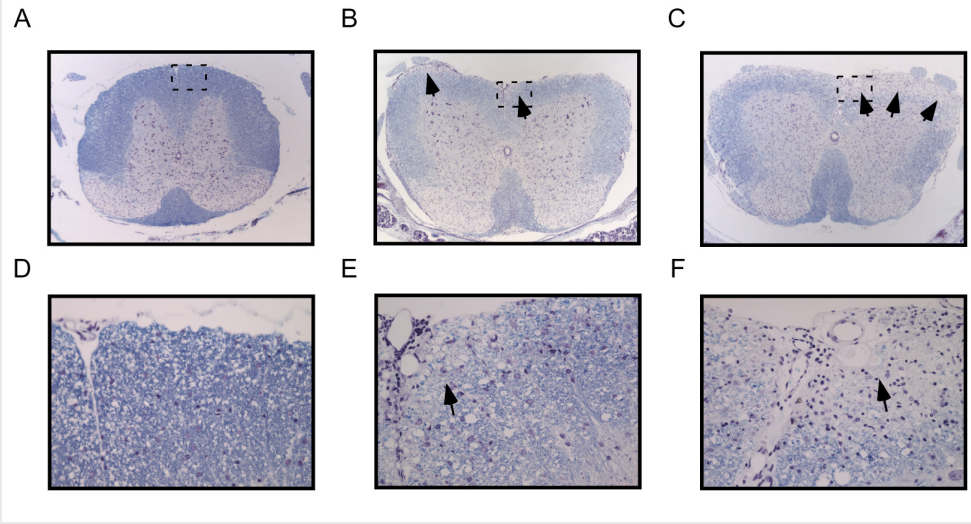

Figure 2.2- HO-1 prevents CNS demyelination. Representative Luxol fast blue staining of spinal cord sections are shown for naïve C57BL/6 mice (**A**) versus C57BL/6 *Hmox-1*$^{+/+}$ (**B**) and *Hmox-1*$^{-/-}$ (**C**) mice, 60 days after EAE induction. Magnifications in A-C are 40x. Dashed rectangles in (A-C) are magnified (400x) in (D-F), respectively. Arrows indicate demyelination.

delayed timing of analysis and the lack of disease relapse (Figure 2.2A-F). We then asked whether induction of HO-1 by CoPPIX, reduced CNS demyelination, an effect that would be consistent with reduction of paralysis (Figure 2.1A-C). Induction of HO-1 after EAE onset in SJL/J mice, prevented CNS demyelination, as compared to ZnPPIX- or vehicle (PBS)-treated controls (Figure 2.3A-C). Taken together, these observations suggest that the ability of HO-1 to revert paralysis during the progression of EAE is associated with inhibition of CNS demyelination.

3.2. HO-1 modulates the effector function of pathogenic T_H cells.

At least two mechanisms could account for the reduction of CNS demyelination and paralysis observed when endogenous HO-1 is expressed (Figures 2.1A and 2.2A-F) or when its expression is induced by CoPPIX (Figures 2.1B,C and 2.3A-C), namely cytoprotection of CNS cells and/or suppression of the immune response leading to CNS injury. While not excluding the first possibility we tested the second one and assessed whether induction of HO-1 suppressed the formation of

"inflammatory foci" in the CNS of mice with previously established EAE. Inhibition of EAE progression and relapse in SJL mice treated with CoPPIX was associated with a 87.2±20% reduction of inflammatory foci in the CNS parenchyma (p=0.0033) and a 62.4±40% reduction of

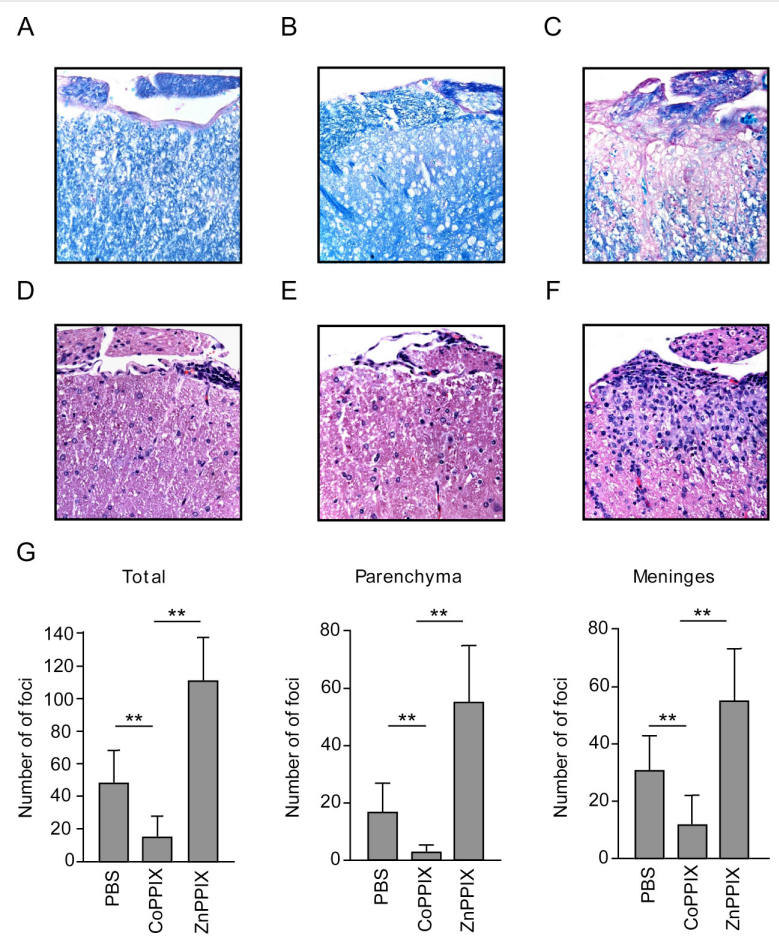

Figure 2.3- Induction of HO-1 prevents CNS demyelination and formation of inflammatory foci. EAE was induced in SJL/mice, randomized two days after disease onset and treated daily with PBS (**A** and **D**), CoPPIX (**B** and **E**) or ZnPPIX (**C** and **F**), as described in Methods. Representative Luxol fast blue (**A** to **C**) and Hematoxylin & Eosin (**D** to **F**) spinal cords staining with adjacent peripheral nerve root are shown, 40 days after disease induction. Magnifications are 160x. Notice some edema of myelin (**A**) and meningeal inflammation (**D**) in PBS treated controls, intact myelin (**B**) and mild meningeal inflammation (**E**) in CoPPIX treated mice and demyelination (**C**) and marked meningeal and parenchymal inflammation (**F**) in ZnPPIX treated mice. (**G**) Number of inflammatory foci were quantified and are illustrated as the mean number of inflammatory foci in the meninges, parenchyma and total (meninges + parenchyma) ± standard deviation (n=8-10 animals per group).

inflammatory foci in the CNS meninges (p=0.013), as compared to vehicle-treated controls, respectively (Figure 2.3D-G). The ratios of inflammatory foci in meninges *versus* parenchyma was

1.75:1 in PBS controls, 5:1 in CoPPIX and 1:1 in ZnPPIX treated mice, suggesting that CoPPIX has a relatively greater effect in reducing inflammatory foci in the parenchyma *versus* the meninges. ZnPPIX, a protoporphyrin which inhibits HO enzymatic activity, increased by 2.3 fold the total number of inflammatory foci (i.e. parenchyma and meninges), as compared to vehicle-treated controls (p=0.0003), suggesting again that endogenous HO-1 activity counters neuroinflammation associated with EAE progression (Figure 2.3D-G). This effect was however, not reflected by an increase in the clinical scores of the disease (Figure 2.1C).

The ability of HO-1 induction to reduce the formation of inflammatory foci in the CNS (Figure 2.3G) was paralleled by a similar reduction in the total number of leukocytes detected within the CNS, as assessed by flow cytometry. Significant numbers of leukocytes ($CD45^{high}$) were detected in the CNS of C57BL/6 mice undergoing EAE (Figure 2.4A) when compared to controls (Figure 2.4B). These were composed primarily of $CD4^+$ T_H cells, $CD8^+$ T cells and $CD45^{high}CD11b^+$ macrophages (Mø) (Figure 2.4B and 2.5A). Induction of HO-1 by CoPPIX decreased by 80±6% the total number of leukocytes ($CD45^{high}$) within the CNS, as compared to PBS-treated controls ($p<0.001$)(Figure 2.5A). This decrease was reflected by an 83±7% decrease in T_H ($p<0.001$) and a 67±17% decrease in $CD8^+$ T cells ($p<0.001$), as compared to PBS-controls (Figure 2.5A). A similar effect was observed for $CD45^{high}CD11b^+$ macrophages (Mø) (*data not shown*). Despite this reduction in the total number of CNS-infiltrating leukocytes, the relative percentage of T_H cells, $CD8^+$ T cells and Mø in the CNS of CoPPIX treated mice was similar to that of PBS or ZnPPIX treated controls (*data not shown).* These data demonstrates that HO-1 induction after EAE

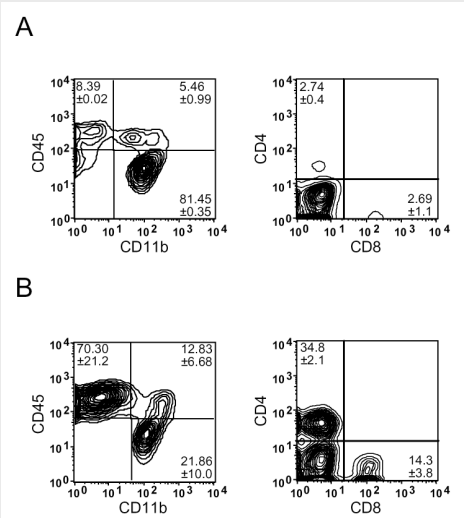

Figure 2.4- CNS leukocyte infiltrates during EAE. Leukocyte infiltrates were analyzed by flow cytometry in (**A**) naïve C57BL/6 mice (n=2) or (**B**) 20 days after immunization (MOG_{35-55} plus CFA). Representative plots are shown with relative percentages of $CD45^+$, $CD11b^+$, $CD4^+$ and $CD8^+$ cells ± standard deviation.

onset decreases very significantly the number of CNS leukocytes and the formation of inflammatory foci within the CNS.

We tested whether HO-1 interfered with the activation, proliferation and/or acquisition of effector function by CNS T_H cells. Induction of HO-1 by CoPPIX reduced by 57±2% the frequency of T_H cells undergoing cell cycle progression ($BrdU^+$) and by 51.5±34% that of T_H cells expressing interleukin-2 (IL-2) in the CNS, as compared to vehicle-treated (PBS) controls (p=0.028 for BrDU

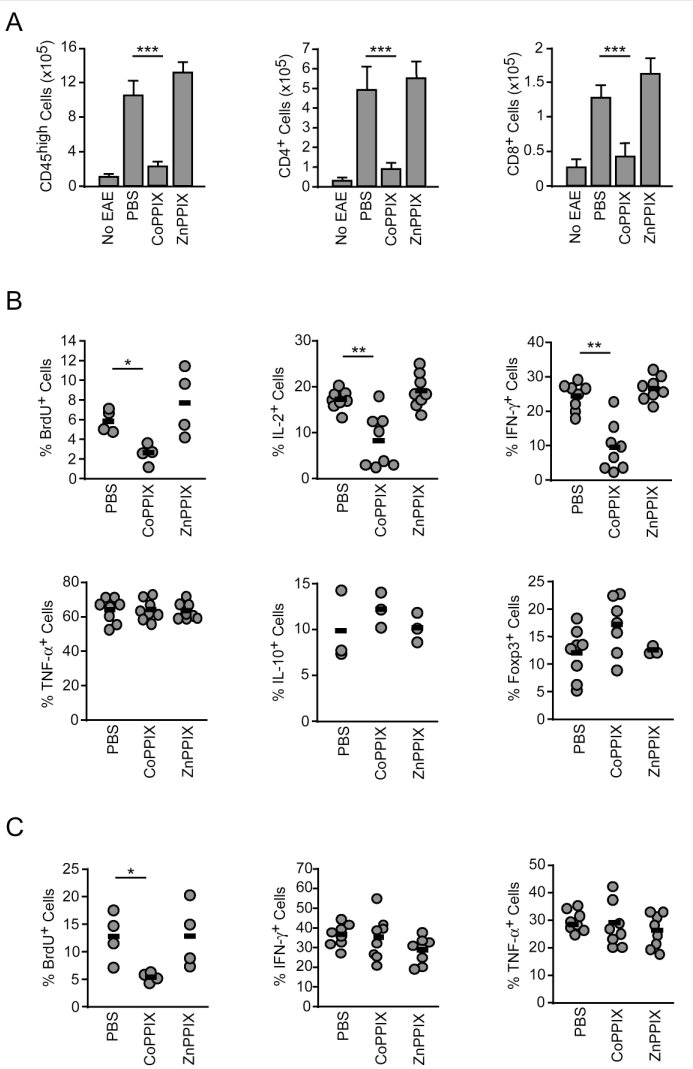

Figure 2.5- Induction of HO-1 reduces the number of T_H cell and $CD8^+$ T cells within the CNS and suppresses T_H cell effector function. EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS, CoPPIX or ZnPPIX, as described in *Methods*. Leukocyte CNS-infiltrates were analyzed by flow cytometry, 20 days post-immunization. (**A**) Number of $CD45^{high}$ (leukocytes), $CD4^+$ T_H cells and $CD8^+$ T cells are shown as mean ± standard deviation (n=4-5 animals per group). The average ± SEM of disease score in PBS, CoPPIX and ZnPPIX treated mice were 2.5±0.3, 0.5±0.3 and 2.25±0.3, respectively (***p<0.001). (**B**) Percentages of CNS $CD4^+$ T_H cells expressing intracellular BrdU (S phase cell cycle progression), IL-2, IFN-γ, TNF-α, IL-10 and Foxp3 (regulatory T cells). (**C**) Percentage of CNS $CD8^+$ T cells expressing intracellular BrdU (cell cycle progression, S phase), IFN-γ, and TNF-α. Each value in (B) and (C) represents an individual animal. Bars indicate mean value of all mice analyzed in each group. (*p<0.05 and **p<0.01).

and p<0.01 for IL-2) (Figure 2.5B). This suggests that induction of HO-1 suppresses T_H cell proliferation within the CNS, an effect that should contribute to suppress EAE progression.

The pathogenic function of CNS-infiltrating T_H cells was also modulated by up-regulation of HO-1. Flow cytometry analysis revealed that HO-1 targeted specifically the expression of interferon-gamma (IFN-γ) in T_H cells. In control-treated mice undergoing EAE there were 24±4% CNS-infiltrating T_H cells expressing intracellular TNF-α and IFN-γ (*data not shown*). Induction of HO-1 by CoPPIX reduced by 60±3% the frequency of T_H cells expressing IFN-γ but failed to affect TNF-α expression in these cells, as compared to PBS-treated controls (Figure 2.5B). Frequency of T_H cells expressing IL-10 (Figure 2.5B) and relative level of IL-10 expression in these cells (*data not shown*) was not modulated by HO-1, an effect that should contribute to suppress EAE progression as well.

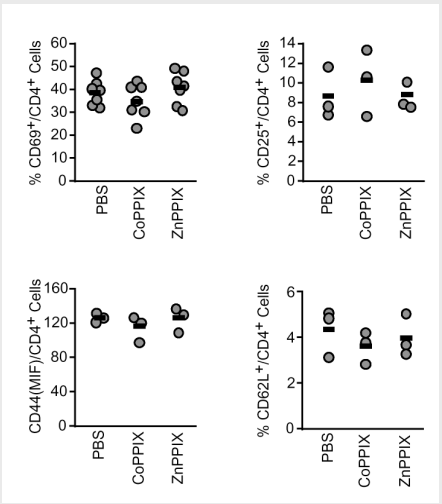

Figure 2.6- Induction of HO-1 does not modulate the expression of activation markers in T_H cells within the CNS. EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS, CoPPIX or ZnPPIX, as described in *Methods*. Leukocyte infiltrates in the CNS were analyzed by flow cytometry, 20 days post-immunization. Each value represents an individual animal. Relative percentage of $CD69^+/CD4^+$ T_H cells, $CD25^+/CD4^+$ T_H cells and $CD62L^+/CD4^+$ T_H cells are shown. For CD44 the mean florescence intensity of CD44 is shown in $CD4^+$ T_H cells. Bars indicate mean value of all mice analyzed under each treatment.

We asked whether the protective effect of HO-1 was associated with modulation of regulatory T cells within the CNS. The frequency of forkhead box P3 (FoxP3)$^+$ regulatory T cells within the CNS was not modulated by HO-1 induction (Figure 2.5B), suggesting that the protective effect of HO-1 does not act via regulatory T cells.

We tested whether expression of T_H cell activation surface markers, including CD69, CD25, CD44 and CD62L were modulated by HO-1 induction. We found that this was not the case for both the frequency of T_H cells expressing these markers (Figure 2.6) and for their relative level of expression (*data not shown*).

Frequency of CNS-infiltrating $CD8^+$ T cells undergoing cell cycle progression ($BrdU^+$) was reduced by 55±21%, when HO-1 was induced, as compared to vehicle-treated (PBS) controls

(p=0.028)(Figure 2.5C), an effect that should contribute to suppress EAE progression[2-4]. Induction of HO-1 did not modulate IFN-γ or TNF-α expression by CNS-infiltrating CD8+ T cells (Figure 2.5C).

3.3. HO-1 and CO suppress myelin-reactive T_H cell re-activation

We hypothesized that HO-1 induction might interfere with re-activation of primed myelin-reactive T_H cells, such as this occurs in the CNS during the development of EAE and presumably also occurs in MS patients. Myelin-reactive T_H cell proliferation was analyzed *in vitro*, upon re-challenge of draining lymph node cells, seven days after immunization with MOG_{35-55}. Induction of HO-1 by CoPPIX reduced myelin-reactive T_H cell proliferation by 60.5±3%, as compared to vehicle- (PBS) or ZnPPIX-treated controls (p<0.001) (Figure 2.7A). The anti-proliferative effect of HO-1 was not due to a reduction of T_H cell numbers in draining lymph nodes (*data not shown*). Moreover, HO-1 induction did not affect the proliferative response of draining lymph node T_H cells to concanavalin A

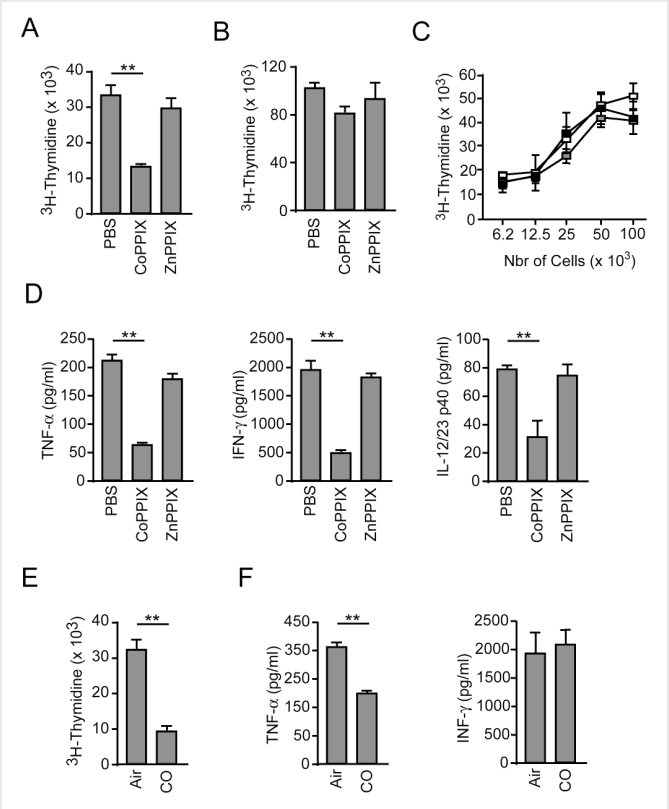

Figure 2.7- HO-1 inhibits the proliferation of T_H cells. Results shown in all panels are mean ± standard deviation from triplicate samples in one out of at least three independent assays, except for IL-12/23 (p40) (one assay)(**p<0.01). (**A**) C57BL/6 mice were treated as described in *Methods*. Eight days after immunization, proliferation of myelin-reactive T_H cell was assessed in vitro, 72 hours after addition of MOG_{35-55} (10 μg/ml). (**B**) C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and treated daily with PBS (n=3), CoPPIX (n=3) or ZnPPIX (n=3). Lymph node cells were isolated 8 days post-immunization. T_H cell proliferation was measured in vitro by ^{3}H-Thymidine incorporation, 72 hours after addition of concanavalin A (2 μg/ml). Results shown are the mean ± standard deviation of one representative assay out of five. (**C**) Increasing numbers of T_H cells (>98% CD4$^+$ T cells) from PBS- (□) (n=3), CoPPIX- (■) (n=3) or ZnPPIX- (■) (n=3) treated mice were co-cultured with T_H cell-depleted lymph node cells (<98% CD4+ T cells) from immunized but otherwise untreated mice. MOG_{35-55}-specific T_H cell proliferation was measured as in (A). Results shown are the mean ± standard deviation. (**D**) Cytokines were assayed in the cell culture supernatants (MOG_{35-55} 100 μg/ml; 72h). (**E**) C57BL/6 mice were exposed to air (n=3) or CO (450 ppm) (n=3), starting two days prior to immunization. T_H cell proliferation was assayed as in (A). (**F**) Mice were treated as in (E) and cytokines were assayed as in (D).

(ConA) (Figure 2.7B). To assess whether suppression of T_H cell re-activation *in vitro* was due to defective priming of naïve myelin-reactive T_H cells *in vivo*, draining lymph node T_H cells (>98% $CD4^+$ T_H cells) were isolated seven days after immunization and re-challenged *in vitro* with MOG_{35-55} in the presence of naïve APC (<2% $CD4^+$ draining lymph node cells). Proliferation of myelin-reactive T_H cells from CoPPIX-treated mice was similar to that of control mice (Figure 2.7C), suggesting that HO-1 induction did not interfere with priming of naïve myelin-reactive T_H cells *in vivo*.

In the same experimental system, induction of HO-1 suppressed TNF-α secretion by 64±2% ($p<0.001$), IFN-γ by 75±2% ($p<0.001$) and IL-12/23(p40) by 61 ±16% ($p<0.01$), as compared to controls (Figure 2.7D). This suggests that HO-1 suppresses the differentiation of primed myelin-reactive T_H cells towards a pathogenic effector phenotype, characterized by the secretion of pro-inflammatory cytokines.

Exposure of C57BL/6 mice to CO (450 ppm), starting two days prior to immunization and continuously thereafter, inhibited by 71±5% the proliferative response of myelin-reactive T_H cells, as compared to control mice exposed to air under similar flow conditions ($p=0.0016$) (Figure 2.7E). In the same experimental setting, CO (450 ppm) inhibited TNF-α secretion by 40±2% ($p=0.0029$) but failed to inhibit IFN-γ secretion (Figure 2.7F), as compared to air-treated controls. This suggests that the immunomodulatory effect of CO may be more restricted than that of HO-1 induction.

3.4. HO-1 expression in APC inhibits myelin-reactive T_H cell proliferation

We reasoned that inhibition of T_H cell proliferation could result from induction of HO-1 expression in T_H cells[22,23] and/or in APCs[24]. To test the first possibility, T_H cells were purified (>98% $CD4^+$ T cells) from MOG_{35-55} immunized mice in which HO-1 was induced by CoPPIX. Proliferation of MOG_{35-55}-reactive T_H cells was monitored *in vitro* in the presence of APC (<2% $CD4^+$ draining lymph node cells) isolated from immunized but otherwise untreated mice. Proliferation of T_H cells from CoPPIX-treated mice was not significantly inhibited, as compared to PBS or ZnPPIX controls (Figure 2.8A). This suggests that HO-1 expression in T_H cells is not sufficient *per se* to suppress T_H cell proliferation. That the anti-proliferative effect of HO-1 is exerted via APC is suggested by the following set of observations. Proliferation of myelin-reactive T_H cells (>98% $CD4^+$ T cells) from

immunized but otherwise untreated mice, was inhibited by 65±7% in the presence of APC (<2% $CD4^+$ draining lymph node cells) from immunized mice treated with CoPPIX, as compared to control APC isolated from PBS- or ZnPPIX-treated immunized controls ($p<0.001$) (Figure 2.8B). T_H

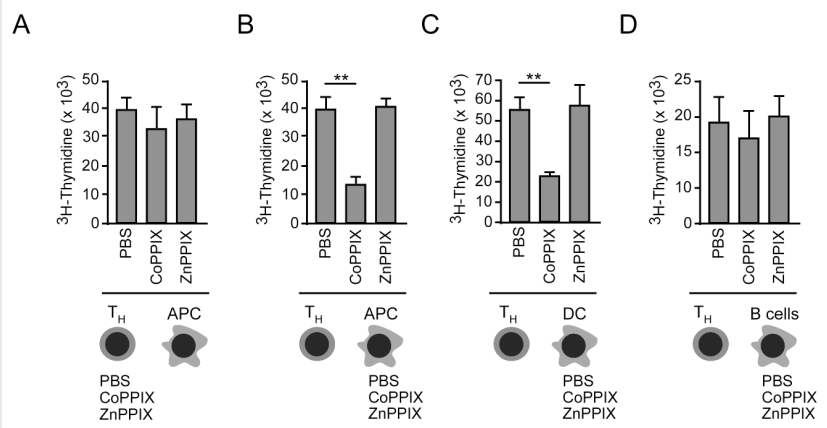

Figure 2.8- The anti-proliferative effect of HO-1 is exerted via APC. Results shown in all panels are mean ± standard deviation from triplicate samples in one out of at least three independent assays (**p<0.01). (**A**) C57BL/6 mice were treated as described in *Methods*. T_H cells (>98% CD4+ cells) from PBS- (n=5), CoPPIX- (n=5) or ZnPPIX- (n=5) treated mice were co-cultured with APC (<98% $CD4^+$ cells) from immunized but otherwise untreated mice. T_H cell proliferation was measured in vitro by ^{3}H-Thymidine incorporation 72 hours after. T_H cells from untreated immunized mice were co-cultured with (**B**) APC (<2% $CD4^+$), (**C**) DC (>98% $CD11c^+$ cells) or (**D**) B cells (>98% $B220^+$ cells) from PBS- (n=5), CoPPIX- (n=5) or ZnPPIX- (n=5) treated immunized mice. T_H cell proliferation was measured as in (A).

cell proliferation was inhibited by 59±3% ($p<0.01$) by DC (>98% $CD11c^+$ cells) (Figure 2.8C) but not by B cells (>98% $CD19^+$ cells) (Figure 2.8D) isolated from CoPPIX treated immunized mice, as compared to DC or B cells isolated from control PBS- or ZnPPIX-immunized mice, respectively. This data suggests that DC expressing HO-1 suppress the proliferation of myelin-reactive T_H cells.

We confirmed that CoPPIX induced the expression of HO-1 in DC *in vivo* and *in vitro*. Administration of CoPPIX to C57BL/6 mice, after EAE onset, induced high levels of HO-1 expression in CNS-infiltrating DC ($CD11c^+$), as compared to PBS- or ZnPPIX-treated controls (Figure 2.9A). Similar effects were observed *in vitro* in that CoPPIX induced a 64 fold increase in HO-1 mRNA expression in bone marrow-derived DC (~80% $CD11c^+$ cells), as compared to vehicle treated controls (Figure 2.9B). This effect was dose-dependent in that the higher the CoPPIX concentration, the higher the expression of HO-1 mRNA (Figure 2.9B). While HO-1 mRNA expression was also induced by ZnPPIX, this effect was significantly less pronounced to that of CoPPIX (Figure 2.9B). Moreover, no dose-dependent effect was observed (Figure 2.9B). These

observations are consistent with the notion that ZnPPIX can induce HO-1 expression moderately while inhibiting its activity strongly, thus acting as a potent HO-1 inhibitor[25]. In the same experimental setting, CoPPIX induced HO-1 protein expression while ZnPPIX failed to do so (Figure 2.9B). To ensure that CoPPIX induced HO-1 expression specifically in DC and not in a putative contaminating cell population, bone marrow-derived DC were further purified (>98% CD11c+ cells). That CoPPIX induced HO-1 expression specifically in CD11c$^+$ cells was shown by immunocytochemistry (Figure 2.9C) and western blot (Figure 2.9D). This data demonstrates that CoPPIX, but not ZnPPIX, induces HO-1 expression in DC, a finding concordant with those of others[24].

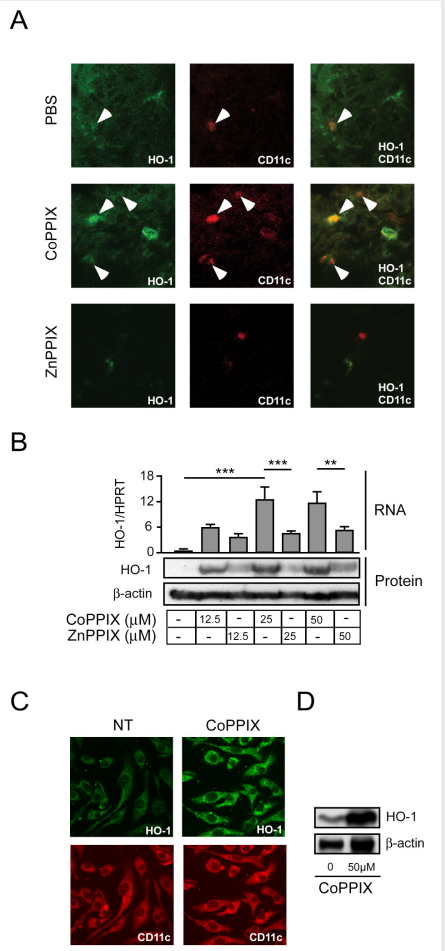

Figure 2.9- CoPPIX induces HO-1 expression in DC *in vivo* and in vitro. (**A**) EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS, CoPPIX or ZnPPIX, as described in *Methods*. HO-1 and CD11c (DC) expression in spinal cord were detected by immunocytochemistry (7 days after beginning of the treatments) and analyzed by confocal microscopy, as described in *Methods*. Shown are HO-1 (green, left panels), CD11c (red, middle panels) and CD11c plus HO-1 (green and red merged, right panels). Magnifications are 240x. Arrows (white) indicate positive staining. (**B**) Unsorted bone marrow derived DC (~80% CD11c$^+$ cells) were exposed to CoPPIX or ZnPPIX and HO-1 mRNA or protein expression were assessed by qRT-PCR and western blotting, respectively. HO-1 mRNA is shown as mean number of HO-1 per HPRT mRNA molecules ± standard deviation (n=3 animals per group). (**C**) Bone marrow derived DC were purified (>98% CD11c$^+$ cells) and exposed to CoPPIX (16h, 50μM), as in (B). HO-1 (green) and CD11c (red) were detected as in (A). Magnification is 400x. (**D**). Expression of HO-1 was detected by western blot in purified DC shown in (E).

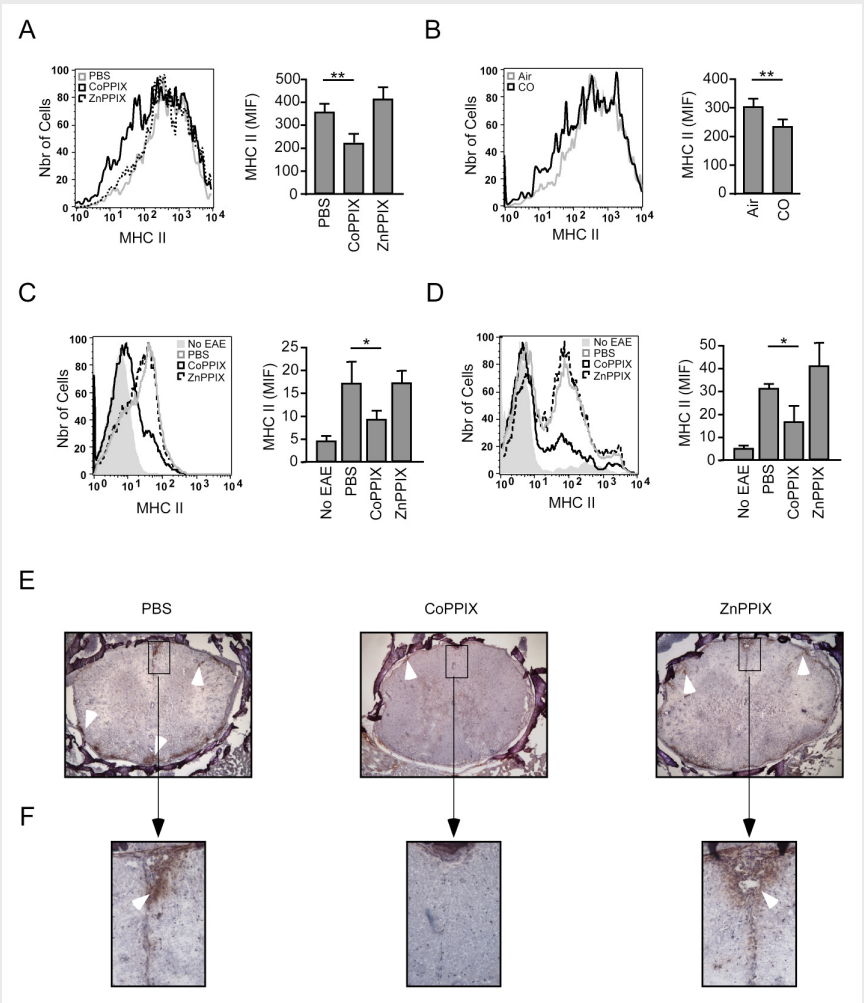

Figure 2.10- Induction of HO-1 and exposure to CO inhibit MHC class II expression in APC. (**A**) C57BL/6 mice were treated daily with PBS (n=4), CoPPIX (n=6) or ZnPPIX (n=6), starting two days prior to footpad immunization (MOG_{35-55} in CFA). Draining lymph node cells were isolated and surface MHC class II expression analyzed in DC ($CD11c^+$) by flow cytometry, eight days post-immunization. Representative histograms and quantifications (mean intensity of fluorescence; MIF) are shown as mean ± standard deviation. **p<0.01. (**B**) C57BL/6 mice were exposed to air (n=6) or CO (450 ppm) (n=7), starting two days prior to immunization and continuously thereafter. Draining lymph node cells were isolated and surface MHC class II expression was assessed in DC ($CD11c^+$) as in (A). Representative histograms and quantification (mean intensity of fluorescence; MIF) are shown as mean ± standard deviation. **p<0.01. (**C** and **D**) EAE was induced in C57BL/6 mice, randomized two days after disease onset and treated daily with PBS (n=9), CoPPIX (n=9) or ZnPPIX (n=9), as described in *Methods*. MHC class II expression in (**C**) microglia ($CD11b^+CD45^{low}$) and (**D**) CNS-infiltrating Mø ($CD11b^+CD45^{high}$) was analyzed by flow cytometry, 20 days post-immunization when controls, i.e. ZnPPIX or PBS, reached maximal disease severity. Representative histograms and quantifications (mean intensity of fluorescence; MIF) are shown as mean ± standard deviation. *p<0.05. (**E-F**) EAE induction and treatments were performed as in (C and D). MHC class II expression was detected by immunocytochemistry and counterstained, as described in *Methods*. Magnifications are 10x (E) and 40x (F). Arrows (white) indicate MHC class II expression.

3.5. HO-1 suppresses MHC class II expression in activated APC

To evaluate further the impact of HO-1 in APC function, draining lymph node DC from immunized C57BL/6 mice were analyzed by flow cytometry. Induction of HO-1 by CoPPIX reduced by 39±12% MHC class II expression in DC (CD11c+), as compared to controls ($p<0.001$) (Figure 2.10A). This effect was specific to MHC class II, as expression of CD40, CD80 and CD86 were not significantly affected (Figure 2.11A). Induction of HO-1 did not alter the frequency or total number of DC in the draining lymph nodes of immunized mice (*data not shown*). Exogenous CO (450 ppm) inhibited by 24±8% MHC class II expression in DC, as compared to air treated controls ($p=0.0011$)(Figure 2.10B). This effect was again specific to MHC class II, as CO failed to inhibit the expression of CD40, CD80 or CD86 in DC (Figure 2.11B).

To test whether HO-1 and/or CO would affect other APC populations involved in the pathogenesis of EAE[26,27], MHC class II expression was analyzed in microglia (CD45low CD11b+) and CNS-infiltrating Mø (CD45high CD11b+) of C57BL/6 mice with established EAE. Induction of HO-1, starting two days after disease onset (~40% EAE incidence), reduced MHC class II expression by 44±13% in microglia (Figure 2.10C) and by 47±24% in CNS-infiltrating Mø (Figure 2.10D), as compared to controls ($p<0.05$ for comparisons of both cell populations to controls).

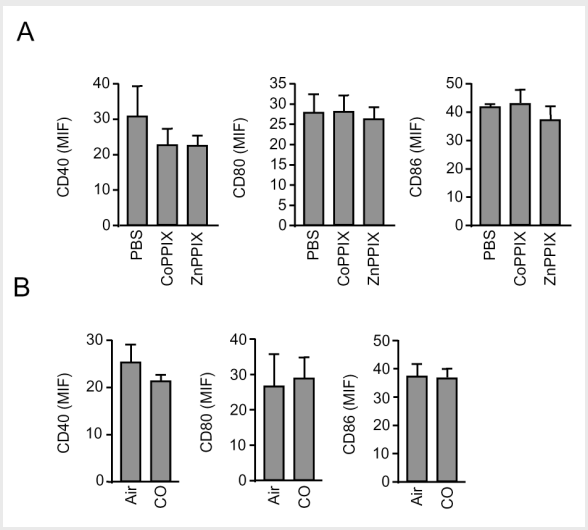

Figure 2.11- Induction of HO-1 does not modulate the expression of co-stimulatory molecules in APC. C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and (**A**) treated daily with PBS (n=4), CoPPIX (n=6), ZnPPIX (n=6), (**B**) exposed to air (n=6) or CO (n=7), as described in *Methods*. Draining lymph node cells were isolated 8 days after immunization and CD40, CD80 or CD86 surface expression in DC ($CD11c^+$) was analyzed by flow cytometry. Quantifications (mean intensity of fluorescence; MIF) are shown as mean ± standard deviation.

MHC class II expressing cells in the CNS of mice with established EAE was confined to the perivascular area and associated primarily with CNS-infiltrating leukocytes, i.e. Mø (Figure 2.10E-F). Low level MHC class II expression was also detected in the parenchyma,

presumably associated with microglia (*data not shown*). Induction of HO-1 by CoPPIX reduced MHC class II expression in both perivascular leukocytes and microglia (Figure 2.10E-F), confirming similar observations made by flow cytometry, used hereby as a quantitative read-out to assess the level of surface MHC class II expression in these cell populations (Figure 2.10C-D).

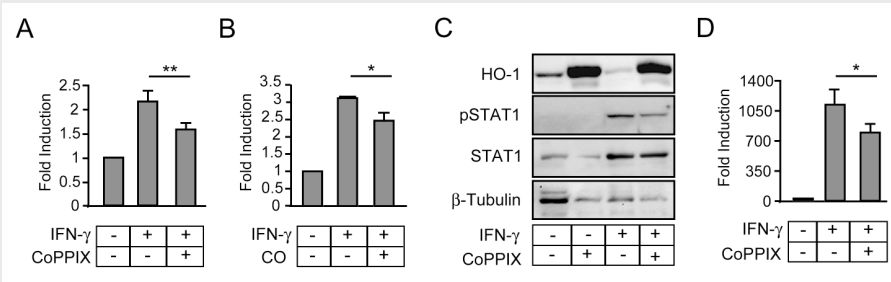

Figure 2.12- HO-1 inhibits STAT-1 phosphorylation and CIITA expression in CNS APC. (**A** and **B**) Expression of MHC class II in microglial BV2 cells was monitored by flow cytometry. When indicated (+) BV2 cells were exposed to CoPPIX (50 μM) or CO (250 ppm), 6 or 16 hours before IFN-γ stimulation (24h; 50 U/ml), respectively. MHC class II expression is shown as fold induction *versus* untreated cells ± standard deviation (n=3-7). **p<0.01 and *p<0.05. (**C**) Phosphorylation of STAT-1, total STAT-1, HO-1 and β-tubulin were detected by Western blot in BV2 cells, treated as in (A). (**D**) Expression of CIITA mRNA was quantified by real time PCR in BV2 cells treated as in (A). Results are shown as mean fold induction *versus* untreated cells ± standard deviation (n=3). *p<0.05.

To address further the mechanism via which HO-1 suppressed MHC class II expression in CNS APC, we tested whether induction of HO-1 in microglial BV2 cells would inhibit MHC class II expression. When stimulated *in vitro* with IFN-γ, microglial BV2 cells up-regulated the expression of MHC class II (Figure 2.12A-B). Induction of HO-1 by CoPPIX inhibited by 27±7% MHC class II expression, as compared to controls ($p<0.05$)(Figure 2.12A). Exposure to CO (250 ppm) mimicked this effect, inhibiting by 20±7% MHC class II expression, as compared to air-treated controls ($p<0.05$)(Figure 2.12B). Induction of HO-1 or exposure to CO had no effect on CD40, CD80 or CD86 expression in BV2 cells (*data not shown*). Induction of HO-1 inhibited IFN-γ-driven phosphorylation of signal transducer and activator of transcription 1 (STAT-1)(Figure 2.12C), without affecting STAT-1 expression (Figure 2.12C). Induction of MHC class II transcription activator (CIITA) mRNA expression by IFN-γ was inhibited by 30±11%, as compared to controls ($p<0.05$) (Figure 2.12D). Given the central role of STAT-1 phosphorylation and CIITA expression in the transcriptional regulation MHC class II expression in APC[28] (*reviewed in*[29]), the inhibitory effect of HO-1 over MHC II expression in these cells might be explained by its ability to suppress STAT-1 phosphorylation and CIITA expression.

4. Discussion

There are several genes that can limit the deleterious effects of inflammation and thus counter the pathogenesis of inflammatory diseases[5,7]. We refer to those genes as "protective genes"[7] and hypothesized that the pathologic outcome of EAE, a prototypical inflammatory disease and well-established model of MS, might be controlled via the expression of the protective gene HO-1. We provide evidence that when expressed endogenously in the context of EAE progression, HO-1 suppresses both severity and mortality associated with the development of this neuroinflammatory disease (Figure 2.1A and Table 2.1). We also show that up-regulation of HO-1 by the administration of CoPPIX after the onset of clinical disease can act therapeutically to reverse paralysis (Figure 2.1B-C and Table 2.2). That the therapeutic effect of CoPPIX is exerted via the induction of HO-1 is unequivocally demonstrated by the observation that i) CoPPIX induces the expression of HO-1 in the CNS of mice undergoing EAE (Figure 2.1D) and ii) the protective effect of CoPPIX is abrogated in *Hmox1*$^{-/-}$ mice (Figure 2.1E). This study represents the second report in which HO-1 induction by the administration of a protoporphyrin after disease onset can reverse disease progression[30].

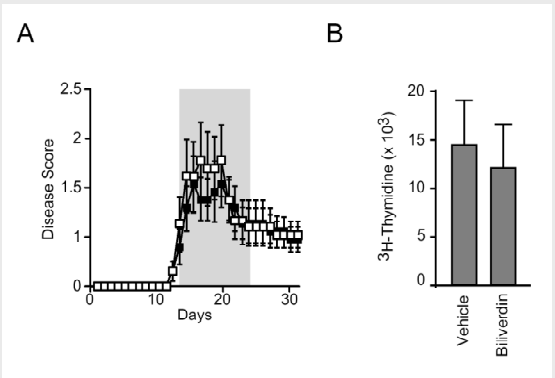

Figure 2.13- Biliverdin does not suppress EAE progression nor does it suppress the proliferation of myelin-reactive T_H cells. (**A**) EAE was induced and disease severity scored, as described in *Methods*. Mice were randomized two days after disease onset and treated daily with biliverdin (■)(n=15) or vehicle (□)(n=15) for the period indicated by the shaded area. Daily clinical scores are shown as mean ± standard error of mean. (**B**) C57BL/6 mice were immunized in the footpad with MOG_{35-55} plus CFA and treated daily with biliverdin (n=3) or vehicle (n=3), starting two days prior to immunization. Draining lymph node cells were isolated 8 days post-immunization and myelin-reactive T_H cell proliferation was assessed in vitro by ^{3}H-thymidine incorporation, 72 hours after addition of MOG_{35-55} (10 μg/ml). Results shown are the mean ± standard deviation from one assay with five independently treated animals per group.

Since exposure of wild type *Hmox1*$^{+/+}$ mice to CO mimicked the protective effects of HO-1 induction by CoPPIX (Figures 2.1F, 2.7E, 2.7F, 2.10B and 2.12B), it is reasonable to assume that when generated via the degradation of heme by HO-1, CO contributes in a significant manner to mediate the protective effects of HO-1. One would expect, however, that the other end-products of heme degradation by HO-1, including biliverdin, which we have

shown to suppress the effector function of alloreactive T_H cells[31], may act in a similar manner. However, our present data suggests that biliverdin does not suppress myelin-reactive T_H cell proliferation nor does it suppress EAE progression (Figure 2.13). One possible explanation may be that the protective effect of HO-1 is exerted within the CNS which biliverdin cannot enter because of its exclusion by the blood brain barrier. While this may explain the lack of EAE suppression afforded by exogenously administered biliverdin it does not explain the apparent inability of biliverdin to suppress the proliferation of myelin reactive T_H cells (Figure 2.13).

The protective effect of HO-1 expression within the CNS might also operate via the reduction of cellular labile Fe^{++}, which can promote the generation of reactive oxygen species through the Fenton reaction[32], as suggested by others[33].

Our data suggests that the mechanism underlying the protective effect of HO-1 is associated with a profound inhibition of leukocyte accumulation (Figures 2.3G and 2.5A) and re-activation (Figure 2.5B-C) within the CNS. This is suggested by the observation that HO-1 induction by CoPPIX administration after EAE onset reduces the number of inflammatory foci (Figure 2.3G) as well as the total number of CNS T_H cells, $CD8^+$ T cells and Mø (Figure 2.5A) and in addition suppresses the proliferation of the remaining T_H and $CD8^+$ T cells (Figure 2.5B-C) infiltrating the CNS.

The anti-proliferative effect of HO-1 was shown *in vitro* to be strictly dependent on DC (Figure 2.8C) that express high levels of HO-1 (Figure 2.9B-C). That a similar effect may occur *in vivo* to arrest EAE progression is suggested by the observation that DC expressing high levels of HO-1 are detected in the CNS of CoPPIX-treated mice in which EAE progression was arrested (Figure 2.9A).

Induction of HO-1 leads to specific inhibition of MHC class II in APC, including DC (Figure 2.10A-B), microglia (Figure 2.10C) and CNS-infiltrating Mø (Figure 2.10D). Induction of HO-1 expression in microglia suppressed STAT-1 phosphorylation as well as CIITA expression, two critical events in the mechanism leading to MHC class II expression in APC[28](*reviewed in*[29]), as well as in the re-activation of myelin-reactive T_H cells in the CNS[34]. This effect is likely to contribute to the overall protective effect of HO-1 induction as MHC class II expression in microglia is thought top be involved in EAE pathogenesis and progression (reviewed in[35]).

That inhibition of MHC class II accounts for the protective effect of HO-1 is supported by the association between certain human MHC class II locus alleles and MS susceptibility (*reviewed in*[36]). Moreover, peptides that interfere directly with MHC class II-mediated antigen presentation to myelin-reactive T_H cells are very efficient in arresting EAE progression[37](*reviewed in*[2]), suggesting again that inhibition of MHC class II expression by APC is probably sufficient *per se* to explain the protective effects of HO-1 observed hereby. We cannot exclude however, that HO-1 might have additional effects that could act in APC or other cell types to suppress the activation and proliferation of myelin-reactive T_H cells. One possibility would be that HO-1 promotes regulatory T cell function[38]. However, such a mechanism can probably be excluded since the number and function of regulatory T cells is unaffected in *Hmox-1*$^{-/-}$ mice, as compared to *Hmox-1*$^{+/+}$ controls, suggesting that endogenous HO-1 expression does not influence regulatory T cell function *(S. Zelenay, A. Chora, M. P. Soares and J. Demengeot, unpublished observation).* Furthermore, we have also observed that despite its ability to suppress ongoing EAE, induction of HO-1 expression failed to modulate the numbers of CNS-infiltrating regulatory T cells (Figure 2.5B).

That HO-1 exerts its protective effects via APCs, including CNS microglia, (Figure 2.10C-D) is relevant for its mechanism of action if one considers that immunomodulation within the CNS is probably required to arrest MS progression. This may explain the relative lack of efficiency in treating MS by controlling exclusively "peripheral" antigen presentation, which is probably not as relevant, whereas prevention of effector T_H cell re-activation by CNS APC almost certainly is[26,34]. Our finding that induction of HO-1, after EAE onset, can suppress T_H cell re-activation and effector function within the CNS (Figure 2.5B) may explain why this approach is effective in suppressing disease progression (Figure 2.1B-C, Table 2.2).

Our present data suggest that upon induction of HO-1 in APC, the effector function of myelin-reactive T_H cells in the CNS is modulated in a manner that suppresses their pathogenicity. This is supported by the observation that induction of HO-1 suppressed the expression of neuroinflammatory, i.e. IFN-γ, but not neuroprotective, i.e. IL-10[39] and TNF-α[40] cytokines in CNS-infiltrating T_H cells (Figure 2.5B). It should be noted, however, that while inhibition of IFN-γ expression in CNS-infiltrating T_H cells is likely to contribute to EAE regression[41], this remains to be formally established as EAE is exacerbated in IFN-γ-deficient mice[42].

One possibility not excluded by the present study is that HO-1 may prevent EAE progression not only by immunomodulation but also by its cytoprotective properties[43,44] in the CNS, i.e. of oligodendrocytes or neurons[45]. Such an effect would be consistent with the observed arrest of EAE progression[46] as well as with our previous observation that cytoprotection afforded by HO-1 can prevent the rejection of transplanted organs[47].

Even with the necessary caution for extrapolating from EAE to MS, there are several independent lines of evidence, which suggest that HO-1 expression affects the clinical outcome of MS. First, HO-1 is expressed in the CNS of MS patients[9]. Second, HO-1 prevents the deleterious effects of inflammation in humans[48]. Third, there is a $(GT)_n$ microsatellite polymorphism in the human *HMOX-1* promoter that controls quantitative aspects of HO-1 inducibility and dictates the incidence of several inflammatory diseases (*reviewed in*[49]). Additional studies are needed to determine whether HO-1 functions to prevent MS progression or promote its remission.

In conclusion, we found that HO-1 suppresses the pathologic outcome of autoimmune neuroinflammation, such as associated with the development of EAE in mice. This effect is mediated, at least partially by CO, which acts on APC to inhibit the expression of MHC class II and presumably the reactivation of pathogenic T_H cells within the CNS. We suggest that modulation of HO-1 expression or administration of CO may be a useful therapeutic strategy to treat MS patients.

5. Methods

Animals. C57BL/6 and SJL/J mice were bred and maintained under specific pathogen-free conditions in accordance with guidelines from the Animal User and Institutional Ethical Comities of the Instituto Gulbenkian de Ciência and the Beckman Center for Molecular Medicine, Stanford University. Mice were used between 6 and 8 weeks of age. *Hmox-1*$^{+/-}$ mice (back-crossed 10 generations into the C57BL/6 background) were originally generated by Shaw-Fang Yet[50](Pulmonary and Critical Care Division, Brigham and Women's Hospital, Boston, MA 02115, USA) and maintained by *Hmox-1*$^{+/-}$ mating, as described before[50], yielding 2-3% of *Hmox-1*$^{-/-}$ mice. Offspring was genotyped by PCR. Briefly, genomic DNA was isolated from the tail and amplified using the following primers. 5' TCT TGA CGA GTT CTT CTG AG 3' and 5' ACG AAG TGA CGC CAT CTG T 3'; 5' GGT GAC AGA AGA GGC TAA G 3' and 5' CTG TAA CTC CAC CTC CAA C 3'. PCRs were repeated 3 times before experiments and once more thereafter to ascertain genotypes. Littermate *Hmox-1*$^{+/-}$ and *Hmox-1*$^{+/+}$ mice were used as controls.

Cells and reagents. Microglial BV2 cells were obtained from E. Blasi (University of Modena and Reggio Emilia, Modena, Italy) and were cultured, essentially as described before[51]. Recombinant mouse IFN-□ (Prepotech) was used to induce MHC class II expression in BV2 cells. MOG_{35-55} was synthesized at the Biopolymers Laboratory, Harvard Medical School. $PLP_{139-151}$ was synthesized at PAN Facility, Beckman Center for Molecular and Cellular Medicine. Cobalt-protoporphyrin IX, zinc-protoporphyrin IX (Frontier Scientific Inc.) and biliverdin hydrochloride (ICN Biomedicals Inc.) were dissolved in 0.2N NaOH, neutralized with 0.2N HCl, adjusted to 1 mg/ml (CoPPIX and ZnPPIX) and 10 mM (biliverdin) with distilled water, and sterilized by filtration. Aliquoted stocks were protected from light and stored at –80ºC until use.

Cytokine assays. Cell culture supernatants were used to measure TNF-α, IFN-γ, and IL-12/23(p40) concentration by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's indications (BD Pharmingen, OptEIATM).

CNS leukocyte infiltration. Leukocytes were isolated from the CNS, essentially as described elsewhere[52]. Briefly, C57BL/6 mice with established EAE were perfused with PBS *in toto*, CNS (spinal cord and brain) was collected, homogenized, digested (30 min, 37°C) in Hanks-balanced salt solution (HBSS; Life Technologies) supplemented with 0.2 mg/ml collagenase VIII (Sigma-Aldrich Química), strained (100 mm) (Becton Dickinson), centrifuged (1200 rpm; 10 min.) and leukocytes obtained by a Percoll (Amersham Biosciences AB) gradient. The total number of $CD45^{high}$ cells, $CD11b^{+}$, $CD4^{+}$ and $CD8^{+}$ T cells in the CNS was assessed by flow cytometry, using a fixed number of latex beads (Coulter Corp.) co-acquired with a pre-established volume of the cellular suspensions.

DC. Bone marrows from naive mice were flushed, and single cell suspensions cultured (37ºC; 5% CO_2, 95% humidity) in RPMI 1640 (GibcoBRL), 2 mM L-glutamine (Sigma-Aldrich Química), 100 U/ml penicillin, 100µg/ml streptomycin (all from GibcoBRL) and 1% granulocyte macrophage colony stimulating factor (GM-CSF) conditioned medium. Medium was replaced every 48h until day six. CD11c cells were either purified, as described above or remained not.

EAE induction and protoporphyrin treatment. Briefly, C57BL/6 and SJL/J mice were immunized sub-cutaneously in the flanks with MOG_{35-55} or $PLP_{139-151}$ (200µg), respectively, emulsified in CFA (Difco Laboratories) supplemented with heat-inactivated *Mycobacterium tuberculosis* (400µg)(Difco Laboratories). C57BL/6 mice received *Pertussis* toxin (i.v. 200ng)(Sigma-Aldrich Química) at the time of immunization and 2 days thereafter. Clinical signs of EAE were evaluated daily and scored as follows: 0: normal; 1: limp tail, 2: partial paralysis of one or both hind limbs, 3: complete paralysis of one or both hind limbs, 4: hind limb paralysis and forelimb weakness, 5: moribund or deceased. Protoporphyrins were administered daily (i.p. 200µl, 5mg/kg). Biliverdin was administered at 5µM/kg every 12 hours.

Flow cytometry and antibodies. Surface markers and intracellular cytokines were detected essentially as described elsewhere[52]. Purified anti-mouse CD4 (clone RM4-5), CD8 (clone YTS169.4), CD11b (clone M1/70), CD11c (clone HL3), CD40 (clone 3/23), CD45 (clone 30-F11), CD80 (clone 16-10A1), CD86 (clone GL1), B220 (clone RA3-6B2), $I\text{-}A^{b}$ (clone AF6-120.1), IL-2

(clone JES6-5H4), IL-10 (clone JES5-16E3), TNF-α (clone MP6-XT22) and IFN-γ (clone XMG1.2) (BD Pharmingen) were used. Anti-FcγII/II receptors mAb was prepared in house from hybridoma (2.4G2) culture supernatants. Antibodies were directly conjugated to Phycoerythrin (PE), Allophycocyanin (APC) or Fluorescein Isothiocyanate (FITC).

In vivo Bromodeoxyuridine (BrdU) incorporation. Mice received BrdU (i.p.; 50μg/g of body weight; 4x every 2 hours)(BD, Pharmingen) and were perfused *in toto* with PBS two hours after the last BrdU injection. CNS leukocyte infiltrates were isolated as described above and nuclear BrdU was detected using the FITC-labeled anti-BrdU Flow Kit, according to the manufacturer's indications (Becton Dickinson, Pharmingen).

Leukocyte isolation and purification. Popliteal and inguinal lymph nodes were homogenized into single-cell suspension in calcium and magnesium-free PBS, 2% FCS. $CD4^+$ and $CD11c^+$ cells were purified, using a single-step anti-CD4 (L3T4) and anti-CD11c (N418) MicroBeads, respectively, according to the manufacturer's indications (Miltenyi Biotec). $CD8^+$ or $CD19^+$ cells were purified by a two-step labeling, consisting of FITC-labeled anti-CD8 (SK1) and anti-CD19 (4G7) mAb (Becton Dickinson), followed by anti-FITC-conjugated MicroBeads (Miltenyi Biotec). Cells were separated using a MidiMACS magnetic isolation system equipped with LS+ column according to manufacturer's indications (Miltenyi Biotec). Cell number was calculated using a Newbauer Chamber (Sigma). Cell purity was assessed by flow cytometry.

MOG_{35-55}-reactive T_H cell proliferation. Popliteal and inguinal lymph node cells were isolated from PBS-, CoPPIX- or ZnPPIX-treated animals, 8 days after subcutaneous footpad immunization (MOG_{35-55} plus CFA). Cells were plated in 96-well microtiter plates ($5x10^5$ cells per well) in RPMI 1640 (GibcoBRL), 2 mM L-glutamine (Sigma-Aldrich Química), 100 U/ml penicillin, 100μg/ml streptomycin, 10% FCS, 50 μM 2-mercaptoethanol (2-ME), 10 mM Hepes (all from GibcoBRL), and 1 mM sodium pyruvate (Life Technologies) and exposed to MOG_{35-55} (10 μg/ml) or concanavalin A (2 μg/ml)(Sigma-Aldrich Química) (72 hours, 37ºC; 5% CO_2, 95% humidity). Cell proliferation was assessed by [^{3}H]thymidine (1 μCi per well) (GE Healthcare) incorporation during the last 6 hours

of culture. [^{3}H]thymidine up-take was evaluated in a scintillation counter (Tomtec, Pharmacia and Upjohn)

Immunocytochemistry. Purified bone marrow-derived DC were plated in glass coverslip slides (Paul Marienfeld GmbH & Co) and fixed in acetone (10 min at –20ºC). The following antibodies were used. Rabbit anti-HO-1 polyclonal antibody (clone SPA895, Stressgene), PE–labeled anti-CD11c mAb (clone HL3; BD Pharmingen) and FITC-labeled goat anti-rabbit polyclonal antibody (Sigma). Slides were mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA) and fluorescence detected by confocal microscopy (Leica Spectral TCS-SP2). Images were acquired using Leica confocal software (version 2.61).

Histology. Mice (6-10 per experimental group) were perfused with PBS *in toto* followed by 10% formalin. Brain and spinal cord sections were embedded in paraffin and stained with Hematoxylin and Eosin or with Luxol fast blue stains and inflammatory foci enumerated in meninges and parenchyma as described elsewhere[53].

Immunohistology. Mice (n=3-10) were perfused *in toto* with PBS, spinal cords harvested, embedded in Tissue-Tek OCT (Sakura Finetechnical) and snap-frozen in liquid nitrogen. Spinal cord sections (10 µm) were fixed in acetone (10 min, -20ºC) and antigen detection was performed essentially as described elsewhere[44]. The following antibodies were used. Rabbit anti-HO-1 polyclonal antibody (clone SPA895, Stressgene), biotin-labeled anti-MHC class II mAb (clone AF6-120.1; BD Pharmingen), PE–labeled anti-CD11c mAb (clone HL3; BD Pharmingen), FITC-labeled goat anti-rabbit (Sigma) polyclonal antibody. Horse radish peroxidase (HRP)-conjugated Streptavidin and Vectastain Elite ABC kit were used according to manufacturer's instructions (Vector Laboratories). HRP stainings were revealed using 3,3-diamenobenzidine (Sigma) and tissues were counterstained with Harris Hematoxylin (Sigma). Images were acquired using a Leica (DM.LB2) microscope, equipped with an Evolution MP5.0 color camera (Media Cybernetics) and free-GIMP 22.10 software. Fluorescence staining was detected and processed as described above.

CO exposure. For *in vivo* experiments mice were placed in a plexiglass gastight 60 l capacity chamber and exposed continuously to CO, as described elsewhere[44]. Briefly, 1% CO (Linde Gas) was mixed with air in a stainless steel cylinder to obtain a final concentration of 250-450 ppm. CO was provided continuously at a flow rate of ~12 l/min. For *in vitro* experiments cells at 37 ºC, 95% humidity in a plexiglass gastight 10 l capacity chamber were exposed continuously to CO (250 ppm in air, 5% CO_2) at 2 l/min. In both cases CO concentration was monitored using a CO analyzer (Interscan Corporation). Air controls were maintained in a similar chamber without CO.

Western blots and antibodies. Western blots were performed essentially as described before[43,44], using anti-HO-1 (SPA896; Stressgene), anti-STAT-1 (Upstate), anti-phospho-STAT-1 (Upstate) rabbit polyclonal antibodies, anti-β-tubulin (Sigma) mouse monoclonal antibody or anti-β-actin (Sigma) mouse monoclonal antibody. HRP-labeled goat anti-rabbit (31460; Pierce) or goat anti-mouse (31439; Pierce) polyclonal antibodies were used to detect the primary antibodies. HRP activity was revealed using chemiluminescence (ECL) and images were acquired using a Kodak 440CF image station equipped with Kodak imaging software.

Real time PCR. Total RNA was extracted from CNS, BV2 cells or bone marrow-derived DCs using RNeasy® Protect Mini Kit (Qiagen), according to manufacturer's instructions. Total RNA was reverse transcribed using SuperScriptII RNase H^- reverse transcriptase (Invitrogen) and random hexamer primers (Invitrogen). Reactions were incubated at 70 °C for 10 min, at 37 °C for 50 min and 95 °C for 5 min using a robocycler (Stratagene). HO-1 (forward; 5' TCT CAG GGG GTC AGG TC 3' and reverse: 5' GGA GCG GTG TCT GGG ATG 3') CIITA (forward; 5' CTC TAC CAC CTC TAT GAC C 3' and reverse: 5' GCT TCT GTC CTG CTT CTA C 3') and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (forward; 5' GTT GGA TAC AGG CCA GAC TTT GTT G 3' and reverse: 5' GAT TCA ACC TTG CGC TCA TCT TAG GC 3') PCR products were detected using Light Cycler real-time quantitative PCR (Roche) essentially as described before[22].

Statistical Analysis. For clinical scores, significance was examined by the Mann-Whitney test. For survival of *Hmox-1*$^{-/-}$ *versus Hmox-1*$^{+/+}$ mice, significance was examined by the Log-Rank Test. Fisher's Exact Test was used for analyses of disease remission. All other statistical analyses were performed using a one-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test. $p<0.05$ was considered significant in all tests.

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Chapter 3: Heme Oxygenase-1 is not required for mouse regulatory T cell development and function

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