1. Abstract

CD4 regulatory T cells (T_{reg}) ensure peripheral tolerance to self-antigens and limit the deleterious effects associated with inflammatory and immune responses by mechanisms that remain to be fully understood. The enzyme heme oxygenase-1 (HO-1), through its known anti-inflammatory activity, is a candidate for a functional role in T_{reg} activity. We compared wild type and HO-1 deficient (*Hmox-1*$^{-/-}$) mice in order to assess the role of HO-1 in mouse T_{reg} development and function under physiologic conditions. The frequency of CD25^{+} and Foxp3^{+} T_{reg} was similar in *Hmox-1*$^{-/-}$ and *Hmox-1*$^{+/+}$ mice. More importantly, CD4^{+}CD25^{+} T_{reg} purified from either *Hmox-1*$^{-/-}$ or *Hmox-1*$^{+/+}$ mice were equally efficient in controlling the proliferation *in vitro* and the expansion *in vivo* of CD4^{+}CD25^{-} T cells, whether or not these responder cells expressed HO-1. In addition, induction of expression of HO-1 *in vivo* did not affect T_{reg} suppressor function. As shown before, expression of HO-1 was higher in T_{reg} than in naïve T cells, however, naturally activated Foxp3^{-} T cells displayed equal amount of HO-1 mRNA as T_{reg}. Finally, we conclude that under physiological conditions in mice, T_{reg} development, maintenance and function are independent of HO-1 activity.

2. Introduction

CD4 regulatory T cells (T_{reg}) maintain immunological self-tolerance, dampen protective immune responses to infection and limit the deleterious effects associated with inflammatory reactions[1-3]. The same cells control lymphopenia-induced proliferation and regulate lymphocyte homeostasis[4, 5]. In addition, T_{reg} have also been implicated in the induction of tolerance to transplanted organs[6] and to the fetus[7]. *In vitro* inhibition of T cell proliferation assay reveals the suppressive function of T_{reg} and is often used as a first approach to study T_{reg} functions[8].

The high affinity interleukin-2 (IL-2) receptor (CD25) is the surface marker most frequently used to identify T_{reg}[1-3]. However, CD25 expression is not restricted to T_{reg} as it is transiently expressed in all T cells upon activation. Moreover, a significant proportion of $CD4^+CD25^-$ T cells display regulatory functions as well[4, 9-11]. Recently, the transcription factor forkhead box P3 (Foxp3) was shown to be expressed specifically in murine T_{reg} and to be required for their development and function[12-14], providing a specific intracellular marker to identify T_{reg}. Despite some 10 years of intense research, there is no agreement on the molecules that mediate T_{reg} activity, although several have been shown to correlate with T_{reg} development and/or function. These include the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Transforming Growth Factor beta (TGF-beta), interleukin-10 (IL-10), glucocorticoid-induced TNF receptor (GITR) or CD25 itself (*reviewed in*[15]).

The enzyme HO-1 is encoded by the ubiquitous stress responsive gene *Hmox-1*. Its expression is inducible in most cells upon exposure to a large variety of endogenous and/or xenobiotic agents[16]. As shown in a growing number of experimental models, HO-1 dampens inflammation, limits tissue injury and promotes tissue repair (*reviewed in*[17]). These activities are thought to rely on the degradation of the pro-oxidant heme into carbon monoxide, iron and biliverdin. Moreover, modulation of HO-1 expression and activity affects graft versus host disease[18], tolerance to tissue graft[19-21] and allo-pregnancy[22], indicating that HO-1 down modulates T cell mediated immune responses.

The similarities in the anti-inflammatory functions attributed to T_{reg} and to HO-1 enzymatic activity raised the possibility that HO-1 would be a key mediator of T_{reg} activities. Several studies have already tested this hypothesis through the use of various *in vitro* assays. Thus, application of

exogenous carbon monoxide to murine[23] and human[24] CD4 T cells *in vitro* has been shown to mimic T_{reg} suppressive effects. Moreover, human $CD4^+CD25^+$ cells were reported to constitutively express HO-1[25] and forced expression of Foxp3 induced HO-1 transcription in a human T cell line[26]. Finally, the suppressor activity of human T_{reg} appeared reduced or enhanced when HO-1 expression and activity were induced or repressed, respectively[26]. Whether human and mouse T_{reg} equally require HO-1 expression to exert their suppressor function *in vitro* and whether this requirement is physiologically relevant remained to be assessed[27]. The availability of HO-1 deficient mice on genetic backgrounds classically used to test T_{reg} activities, prompted us to directly assess whether HO-1 acts physiologically to support T_{reg} function in mice. We found that a functional *Hmox-1* allele is not required for the development of T_{reg} or for their ability to control T cell proliferation *in vitro* and *in vivo*. Our findings, therefore, provide no evidence in support of a role for HO-1 in T_{reg} development and function.

3. Results

3.1. Enhanced expression of HO-1 in mouse naturally activated T cells

Previous reports indicated that Foxp3 expression in human T cells induces HO-1 transcription[26], although T cell activation *per se* also promotes HO-1 expression[25]. To assess whether in mouse CD4 lymphocytes HO-1 and Foxp3 expression are correlated, BALB/c CD4 T cells were fractionated according to their expression of the activation markers CD45RB and CD25 and submitted to quantitative real-time RT-PCR (Figure 3.1). As expected, Foxp3 mRNAs were 10-fold more abundant in $CD25^+$ cells than in activated/memory $CD25^-CD45RB^{low}$ cells and barely detectable in naïve $CD25^-CD45RB^{high}$ cells (Figure 3.1A).

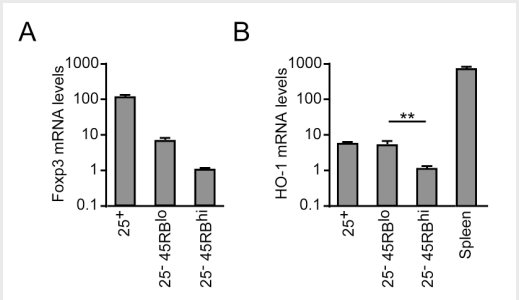

Figure 3.1- Naturally activated CD4 T cells display higher levels of HO-1 mRNA than naïve T cells. Relative Foxp3 (**A**) and HO-1 (**B**) mRNA levels determined by real-time PCR. $CD4^+$ cells, either $CD25^+$ (25^+), $CD25^-CD45RB^{low}$ (25^-45RB^{lo}) or $CD25^-CD45RB^{high}$ (25^-45RB^{hi}) were isolated from pooled spleen and LN of 10 week-old *Hmox-1*$^{+/+}$ BALB/c mice. Total splenocytes served as reference for HO-1 expression. **, $P<0.01$ for 25^-45RB^{hi} versus 25^+ or 25^-45RB^{lo}.

These results are consistent with those obtained after intracellular staining for Foxp3 (*data not shown*). In contrast, both subsets of naturally activated CD4 cells ($CD25^+$ and $CD25^-CD45RB^{low}$) expressed equal amounts of HO-1 mRNA, that was 5-fold higher than that of naïve T cells ($CD25^-CD45RB^{high}$), but 20-fold lower than that of total splenocytes (Figure 3.1B). This analysis reveals that although HO-1 expression correlates with T cells activation status, Foxp3 and HO-1 expression are not directly correlated in murine CD4 T cells.

3.2. *In vivo* induction of HO-1 expression does not affect regulatory T cell function

We next tested whether mouse T_{reg} suppressor activity was enhanced when HO-1 expression was induced above steady-state level. For this purpose, mice were treated twice with CoPPIX, a chemical inducer of HO-1 expression[18], at 24 h intervals. $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ cells were purified 24 h thereafter. As shown previously for total CD4 T cells[21], this regimen induced significant HO-1 overexpression in $CD4^+CD25^+$ cells albeit less than in total splenocytes (Figure

3.2A). When cultured with anti-CD3 and irradiated splenocytes as APC, $CD4^+CD25^+$ cells isolated from CoPPIX-treated and control mice were equally unresponsive to TCR triggering (Figure 3.2B). In cultures containing $CD4^+CD25^-$ cells, T_{reg} cells purified from CoPPIX-treated and control mice suppressed proliferation with similar efficiency, independently of the ratio T_{reg}: $CD4^+CD25^-$ cells (Figure 3.2B). We also monitored whether induction of HO-1 expression affects the responding T cells ($CD4^+CD25^-$) and the APC in similar proliferation assay. In the absence of T_{reg}, $CD4^+CD25^-$ cells proliferative response was not significantly modified whether T cells or APC were isolated from treated or control mice (*data not shown*). Finally, the efficiency of T_{reg} to mediate suppression was tested within each combination of cell subsets and was similar whether T_{reg}, $CD4^+CD25^-$ cells or APC were isolated from CoPPIX-treated or control mice (*data not shown*). These findings indicate that induction of HO-1 expression in mouse T_{reg} does not modify their suppressor activity. *Hmox-1*$^{-/-}$ mice have normal frequency of regulatory T cells.

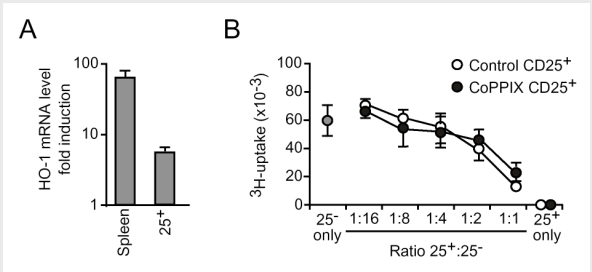

Figure 3.2- Induction of HO-1 expression *in vivo* does not affect $CD4^+CD25^+$ T cell suppressor function. Wild type mice were treated or not with CoPPIX, twice at one day interval and sacrificed 24 hours after the last injection. (**A**) RT-PCR for HO-1 and HPRT mRNA were performed on total splenocytes and $CD4^+CD25^+$ cells isolated from LN of treated and control mice. Shown is the fold induction of HO-1 expression normalized for HPRT in treated versus control cells. (**B**) Suppression of proliferation assay was set with wild type $CD4^+CD25^-$ cell and irradiated splenocytes. Sorted $CD4^+CD25^+$ cells isolated from LN of treated (CoPPIX $CD25^+$) or control (Control $CD25^+$) mice were added to the culture at the indicated ratios.

To directly assess the contribution of HO-1 to mouse T_{reg} function we analyzed the loss of function mutant mice *Hmox-1*$^{-/-}$. Three-month-old BALB/c *Hmox-1*$^{-/-}$ mice produced in our SPF facility did not show obvious abnormalities in body weight,

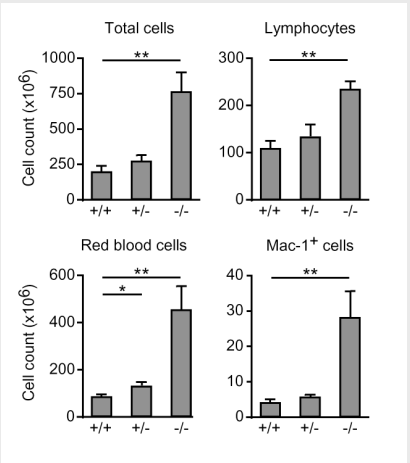

Figure 3.3- Splenomegalia in *Hmox-1*$^{-/-}$ mice. Number of total cells, lymphocytes, RBC and Mac-1^+ cells in the spleen of 3-month-old *Hmox-1*$^{+/+}$ (+/+), *Hmox-1*$^{+/-}$ (+/-) and *Hmox-1*$^{-/-}$ (-/-) BALB/c mice. **, P<0.01 for -/- versus +/+ or +/- and *, P<0.05 for +/- versus +/+. Data represent the average ± SD for each group (n=3). One representative out of two independent experiments.

overall behaviour and posture. However, splenomegalia was clearly noticed upon dissection. Total number of splenocytes was increased on average by a factor of three in *Hmox-1*$^{-/-}$, as compared to *Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ animals (Figure 3.3). Contrary to what has been described for older mice[28], increased cellularity in *Hmox-1*$^{-/-}$ animals was restricted to the spleen and was not noticed in the lymph nodes (LN), bone marrow or thymus (*data not shown*). Flow-cytometry analysis revealed that splenomegalia in *Hmox-1*$^{-/-}$ mice resulted from a proportional accumulation of haematopoietic cells including lymphocytes and red blood cells (Figure 3.3). The total numbers of B cells (B220^{+}, IgM^{+} or IgD^{+}) and T cells (TCR^{+}) were increased by a factor of two in *Hmox-1*$^{-/-}$ *versus Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ animals. The frequency of activated T cells was similar for all genotypes, as assessed by the expression of CD69, CD44 or CD45RB (*data not shown*). The frequency of Mac-1^{+} cells was increased by 2-3 fold in *Hmox-1*$^{-/-}$ (10.7 ± 2.4 %) *versus Hmox-1*$^{+/-}$ (3.8 ± 0.2%) or *Hmox-1*$^{+/+}$ animals (3.6 ± 0.7%), resulting in a more than 5-fold increase in the total number of these cells (Figure 3.3). As reported by others[29], serum IgM but not IgG concentration was increased in *Hmox-1*$^{-/-}$ *versus Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ mice (*data not shown*) while analyses of thymocytes and various LN cell populations did not reveal differences between *Hmox-1*$^{-/-}$, *Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ mice (*data not shown*). The frequency of Ter119^{+} erythrocyte precursors in the bone marrow was decreased by 2-fold in *Hmox-1*$^{-/-}$ *versus Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ mice (*data not shown*). A similar analysis of hmox$^{-/-}$ C57Bl-6 mice indicated milder splenomegalia (2-fold increased cellularity) and no obvious

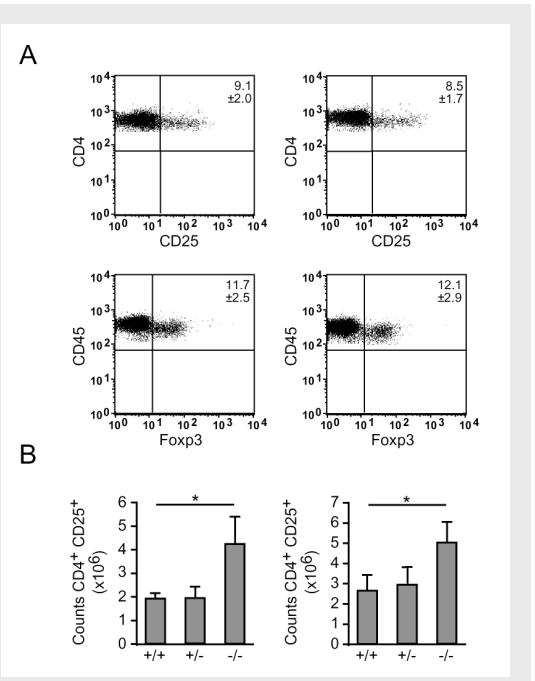

Figure 3.4- ***Hmox-1* deficient mice have normal frequency of regulatory T cells.** (**A**) FACS analysis of splenocytes from 3-month-old *Hmox-1*$^{+/+}$ (+/+, left) and *Hmox-1*$^{-/-}$ (-/-, right) BALB/c mice. Staining for surface CD25 (top) and intracellular Foxp3 (bottom) are shown inside a CD4^{+} gate. (**B**) Number of CD4^{+} CD25^{+} (left) or Foxp3^{+} (right) in the spleen of *Hmox-1*$^{+/+}$, *Hmox-1*$^{+/-}$ and *Hmox-1*$^{-/-}$ mice. *, P<0.05 for -/- versus +/+. Data represent the average ± SD for each group (n=3). One representative out of two independent experiments is shown.

unbalanced distribution of lymphocytes subsets (*data not shown*).

Finally, using either CD25 or Foxp3 to identify T_{reg}, the frequency of T_{reg} in the thymus, spleen or LN was not different in *Hmox-1*$^{-/-}$ mice when compared to *Hmox-1*$^{+/-}$ or *Hmox-1*$^{+/+}$ mice (shown for spleen, Figure 3.4A). Increased splenic cellularity in *Hmox-1*$^{-/-}$ mice was associated with a 2-fold increase in total T_{reg} numbers (Figure 3.4B) similarly to that of conventional CD4 T cells (*data not shown*). These results indicate that HO-1 deficiency does not affect T_{reg} production in the thymus or their maintenance in the periphery.

3.3. Expression of HO-1 by CD4 T cells is not necessary for regulatory T cell suppressor function *in vitro*

The functional capacity of CD4$^+$CD25$^+$ T_{reg} from *Hmox-1*$^{-/-}$ mice was first tested *in vitro* (Figure 3.5). T_{reg} isolated from *Hmox-1*$^{-/-}$ BALB/c mice did not proliferate when cultured in presence of anti-CD3 and irradiated splenocytes (Figure 3.5A). Moreover, wild type CD4$^+$CD25$^-$ cells proliferation was suppressed with the same efficiency irrespectively of whether T_{reg} cells were purified from *Hmox-1*$^{-/-}$ or *Hmox-1*$^{+/+}$ BALB/c mice (Figure 3.5A). In addition, cultures of CD4$^+$CD25$^-$ cells and APC isolated from *Hmox-1*$^{-/-}$ BALB/c

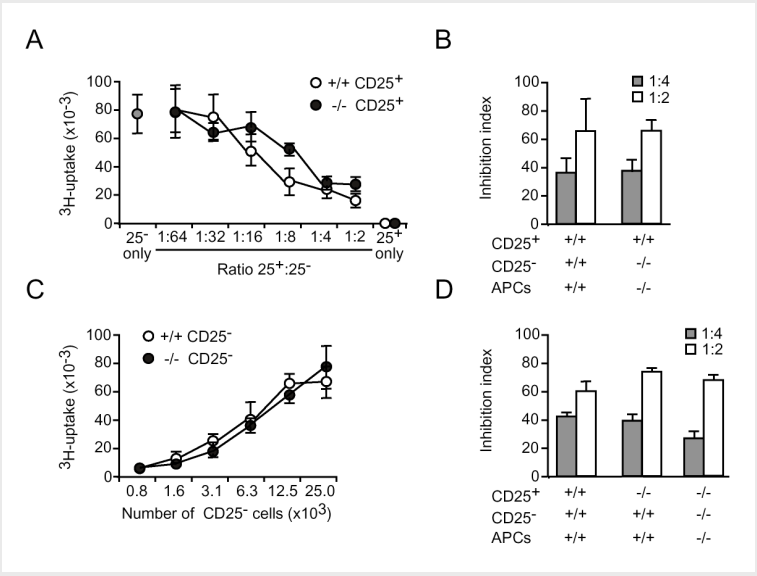

Figure 3.5- CD4$^+$CD25$^+$ and CD25$^-$ cells from *Hmox-1*$^{-/-}$ mice have normal proliferative and suppressive capacity *in vitro*. CD4$^+$ cells either CD25$^-$ or CD25$^+$ were isolated from pooled LN of 3 mice either BALB/c (A-C) or C57BL/6 (D) *Hmox-1*$^{+/+}$ (+/+) or *Hmox-1*$^{-/-}$ (-/-). (**A**) CD4$^+$CD25$^+$ cells from *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$ mice were added at the indicated ratios to a fixed number of *Hmox-1*$^{+/+}$ CD4$^+$CD25$^-$ cells and irradiated splenocytes. (**B**) As in A except that CD4$^+$CD25$^-$ and APC cells were either *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$. Shown is the percentage of inhibition [(cpm in control - cpm in experiment)/cpm in control] in co-cultures set at ratios CD4$^+$CD25$^+$: CD4$^+$CD25$^-$ of 1:4 (Black bars) and 1:2 (white bars). (**C**) CD4$^+$CD25$^-$ cells, either *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$, were plated at the indicated number/well. A-C, one representative result out of three independent experiments is shown. (**D**) As in B except that C57Bl/6 *Hmox-1*$^{+/+}$ and *Hmox-1*$^{-/-}$ mice were used.

mice were equally sensitive to the suppressive effects of T_{reg} (Figure 3.5B) and proliferated equally in absence of T_{reg} (Figure 3.5C). Additional combinations of the three cellular subsets isolated from control or mutant BALB/c mice did not reveal difference in the proliferative responses (*data not shown*). To exclude that the BALB/c genetic background contains genetic elements that specifically compensate the HO-1 requirement of T_{reg} suppressor function, the experiments described above were repeated using *Hmox-1*$^{-/-}$ C57BL/6 mice. Similar suppression indexes were obtained whether T_{reg}, CD4$^+$CD25$^-$ cells or APC were isolated from *Hmox-1*$^{-/-}$ or *Hmox-1*$^{+/+}$ C57BL/6 mice, irrespectively of the co-culture combinations (Figure 3.5D, and *data not shown*). We conclude that HO-1 expression is not necessary for mouse T_{reg} suppressor function *in vitro*.

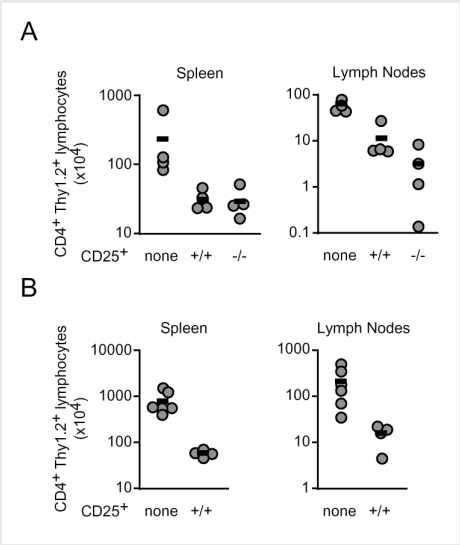

Figure 3.6- CD4$^+$CD25$^+$ cells from *Hmox-1*$^{-/-}$ mice display normal regulatory function *in vivo*. BALB/c SCID mice received 2.5x10^5 CD4$^+$CD25$^-$ cells purified from *Hmox-1*$^{+/+}$ (**A**) or *Hmox-1*$^{-/-}$ (**B**) mice alone or together with an equal number of CD4$^+$CD25$^+$ cells isolated from *Hmox-1*$^{+/+}$ (+/+) or *Hmox-1*$^{-/-}$ (-/-) mice, and analyzed 4 weeks later. Shown is the total number of CD4$^+$Thy1$^+$ lymphocytes recovered from the spleen (left) and pooled LN (right). Each dot represents one mouse. P<0.05 for single transfer versus co-transfers for both spleen and LN (Mann-Whitney test).

3.4. Expression of HO-1 by CD4 cells is dispensable for regulatory T cell activity *in vivo*

To assess whether HO-1 expression by T_{reg} is required for their function *in vivo*, we tested the capacity of *Hmox-1*$^{-/-}$ BALB/c T_{reg} to control lymphopenia induced proliferation. Lymphocyte-deficient BALB/c SCID mice received wild type syngeneic CD4$^+$CD25$^-$ cells either alone or together with an equal number of CD4$^+$CD25$^+$ T_{reg} isolated from either *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$ BALB/c animals. The number of CD4 T cells recovered in spleen and LN was determined four weeks thereafter (Figure 3.6A). When compared to single CD4$^+$CD25$^-$ cell-transfer, co-transfer with either *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$ CD4$^+$CD25$^+$ cells resulted in a similar log reduction in the number of recovered CD4 T cells in the spleen or LN (Figure 3.6A).

We next tested whether the control of lymphopenia-induced expansion by T_{reg} requires the expression of HO-1 in effector T cells. Additional adoptive transfer experiments were performed using $CD4^+CD25^-$ cells purified from *Hmox-1*$^{-/-}$ BALB/c mice and T_{reg} ($CD4^+CD25^+$) from *Hmox-1*$^{+/+}$ BALB/c mice (Figure 3.6B). In this system, T_{reg} activity resulted also in a log reduction in the number of recovered CD4 cells in spleen and LN. Taken together, these findings demonstrate that HO-1 expression by $CD4^+CD25^-$ or $CD4^+CD25^-$ is not required for T_{reg} to exert their function *in vivo.*

4. Discussion

In the present study we explored the possibility that HO-1 expression in murine CD4 cells is necessary for the development and function of regulatory T cells. This hypothesis was driven by the apparent overlap of T_{reg} and HO-1 immuno-regulatory functions in various experimental models. Previous studies using modulators of HO-1 expression and/or activity in human cells *in vitro* concluded that HO-1 expression is necessary for T_{reg} to exert their suppressor function[25, 26]. However, whether these conclusions apply *in vivo* remained to be determined. The recent availability of *Hmox-1*$^{-/-}$ mice on genetic backgrounds classically used to test T_{reg} function (i.e. BALB/c and C57Bl/6) provides the opportunity to address the role of HO-1 for mouse T_{reg} function under physiological conditions. Our conclusions are that HO-1 is not essential for mouse T_{reg} development, maintenance and function.

Consistent with previous expression patterns of CD4 subsets[11], we failed to observe a direct correlation of HO-1 with Foxp3 expression. Further, using conventional drug mediated induction of HO-1 expression, we reveal that elevated level of HO-1 expression in mouse T_{reg} cells does not correlate with increased suppressor efficiency as measured *in vitro*. These findings contrast with previous studies reporting that Foxp3 expression induces HO-1 transcription and that modulation of HO-1 transcription affects T_{reg} activity[25, 26]. The simplest explanation for this discrepancy may be differences in experimental conditions. For example, we analyzed *ex vivo* unmanipulated freshly isolated cells while the previous study was essentially conducted *in vitro*. Perhaps more importantly, we studied LN mouse cells while the previous work addressed human PBLs and modulation of HO-1 expression may affect T_{reg} in a species-specific and/or tissue-specific way.

To further determine whether T_{reg} function requires HO-1 expression, we followed a classical *in vivo* lost of function approach, using *Hmox-1*$^{-/-}$ mice. We found no abnormalities of T_{reg} development or function in these mice. The frequency of T_{reg} in the thymus, spleen and LN was similar in *Hmox-1*$^{+/+}$ and *Hmox-1*$^{-/-}$ mice, indicating that T_{reg} development (thymus) and peripheral maintenance (spleen and LN) are not dependent on HO-1 expression, in either T_{reg} or any other cell type. Unlike null mutations of genes encoding IL-2, IL-2R or Foxp3 that affect the development and/or function of T_{reg}[1], and as described before[28, 30], HO-1 deficiency was not associated with

severe systemic lymphoproliferation early in life. Most likely, cell accumulation in the spleen is a consequence of reduced degradation of the pro-oxidant heme that normally occurs at high rate in this organ[31]. In keeping with the well-established role of T_{reg} in regulating lymphocyte homeostasis, the absence of severe systemic lymphoproliferative disease in young adult HO-1 deficient mice suggested that T_{reg} function was not dramatically reduced in these mice. Regulatory CD4 T cell subsets isolated from *Hmox-1*$^{-/-}$ mice inhibited T cell proliferation equally as wild type T_{reg}, once more suggesting that T_{reg} development and function are independent of expression of HO-1. Noteworthy, the *in vitro* and *in vivo* assays we used to test T_{reg} activity provide both qualitative and quantitative measurements[32] and were totally unable to demonstrate any impact of HO-1 expression in T_{reg} activity. As an alternative test for the possible contribution of HO-1 in an inflammatory model, we tested T_{reg} control of lymphopenia induced proliferation, a process shown to be promoted to a large extent by colonizing microbial flora in addition to *bona fide* homeostatic expansion[33]. Again, we failed to evidence a difference between T_{reg} isolated from wild type or hmox$^{-/-}$ mice and so we can conclude that a functional HO-1 allele in either conventional or regulatory T cells is not essential for mouse T_{reg} activities.

A valid question is whether specific compensatory mechanisms in HO-1-null mutants may mask the otherwise normal contribution of HO-1 to T_{reg} function. In this regard, it is worth noting that the two independent *Hmox-1* knock-out alleles, hmox^{tmKPoss} and hmox^{tmMlee} engineered and published by Poss *et. al.*[28] and Yet *et. al.*[30] respectively, can only be maintained by heterozygote breeding and only 10 to 20% of homozygote embryos develop to term. This characteristic appears independent of the genetic background as Hmox$^{+/Mlee}$ either 129sv/B6, BALB/c or C57Bl/6 ([19, 30] and this work) all yield a similar low frequency of live homozygote progenies. Both hmox^{tmKPoss} and hmox^{tmMlee} homozygote survivors show an absence of detectable HO-1 protein by western blot[28, 30] and live up to one year, independently of their genetic background ([28, 30] and our unpublished results). The cause of early embryonic lethality and the possible compensatory mechanisms that take place in the few *Hmox-1*$^{-/-}$ survivors remain to be established. Whether these early developmental compensatory mechanisms participate also in late lymphocyte physiology is unlikely but cannot be formally excluded. The generation of tissue specific inducible HO-1 knock-out mice will provide the necessary tool to answer this question.

The finding that expression of HO-1 by CD4 T cells is not required for T_{reg} function should not preclude other levels of interactions between HO-1 and T_{reg} activities. For instance, by severely limiting effector T cell number and responses, HO-1 may participate in inverting the effector/T_{reg} ratio in particular regimens of tolerance induction[19]. It is also conceivable that the anti-inflammatory activity of HO-1 in dendritic cells and macrophages may affect the activation and expansion of T_{reg} that normally follows microbial infection[34-36]. Finally, although the progressing inflammatory disease in the *Hmox-1*$^{-/-}$ mice may not be due to a T_{reg} deficiency, increased T_{reg} numbers may delay the disease. The absence of a direct correlation of the two immuno-regulatory pathways controlled by T_{reg} and HO-1 raises the possibility that they may operate independently to ensure a robust control of potentially severe deleterious inflammatory reactions. T_{reg} would function to provide fine tuning of antigen-specific immune responses[3] while HO-1 expression in tissues, monocytes/macrophages and dendritic cells would ensure local return to homeostasis. Testing these hypotheses will await the production of alymphoid *Hmox-1*$^{-/-}$ mice and analysis of chimeras in which HO-1 activity is restricted to specific cellular subsets.

Finally, the possibility that the function of human and mouse T_{reg} would rely on different molecular pathways has multiple consequences, at the fundamental and clinical level. This intriguing issue is out of the scope of the present study, but it is worth noticing that contrary to the genes involved in T_{reg} development and function, such as Foxp3, CTLA-4, IL-2, IL-2 receptor and TGF-beta, polymorphisms in the HO-1 locus have yet not been associated with autoimmune diseases. Moreover, HO-1 gene polymorphisms in donor and not in recipient have been associated with graft survival[37]. Irrespective of these considerations, our findings, indicating that mouse T_{reg} development, maintenance and function do not require HO-1, call for a re-evaluation of the links between the HO-1 and T_{reg} immuno-modulatory pathways.

5. Methods

Mice. Mice were bred and maintained under specific pathogen-free (SPF) conditions at the Instituto Gulbenkian de Ciência animal facility. Experimental protocols were approved by the institutional ethical comity as well as the Portuguese veterinary general division. *Hmox-1*$^{-/-}$ mice in the BALB/c and C57BL/6 backgrounds (back-crossed 10 generations to the respective background) were provided by Shaw-Fang Yet (Brigham and Women Hospital, Harvard Medical School, Boston, MA) and introduced at the SPF facility by embryo transfer. Mice were bred as heterozygotes and yield ~5-10% homozygous offspring[28, 30]. Littermate *Hmox-1*$^{+/+}$ and *Hmox-1*$^{+/-}$ were used as controls. All experiments described were performed with 2 to 3 month-old mice, before the development of inflammatory lesions[30]. BALB/c SCID mice were originally purchased from the Jackson Laboratory (USA).

Antibodies and reagents. Allophycocyanin (APC), Cy-Chrome and Phycoeritrin (PE)-conjugated anti-CD4 mAb (clone RM4-5), CD45RB-PE (16A), and Thy1.2-FITC (53-2.1) were purchased from BD Pharmingen (Sandiego, CA). Anti-Foxp3-PE (FJK-16s) was purchased from e-biosciences (www.ebioscience.com). B220-APC (RA3-6B2), and CD25 (PC61)-AlexaFluor™ 488 were produced at the Instituto Gulbenkian de Ciência. CoPPIX (Frontier Scientific Inc., Logan, UT) was dissolved in 0.2 N NaOH, neutralized with 0.2 N HCl, adjusted to 1 mg/ml in H_2O, and sterilized by filtration. Aliquoted stocks were stored at –80 ºC until use. CoPPIX was administered daily (i.p., 200 µl, 5 mg/kg).

Cell preparation and flow cytometric analysis. Cells were stained as described previously[9]. Briefly, single cell suspensions were obtained from spleen or pooled lymph nodes (axillary, inguinal, braquial and mesenteric) were prepared in PBS 2% FCS; 0.01% sodium azide (FACS buffer). After Ab staining (25 µl Ab mixture, 20' on ice) and washes in FACS buffer, cells were stained with propidium iodide (PI) in FACS buffer. Analyses were performed on a FACSCalibur (BD) using Cell Quest™ software, allowing the exclusion of dead cells (PI^+) inside the indicated gates. Total number of cell counts was deduced from the acquisition of a fixed number of 10 µm latex beads (Coulter Corp. Miami, FL) mixed with a known volume of unstained cell suspension.

Cell purification and adoptive transfer. Pooled lymph nodes or splenocytes stained with anti-CD4-PE and CD25-Alexa mAbs or with anti-CD4-Cy-Chrome, CD25-Alexa and CD45RB-PE were purified using a MoFlo high speed cell sorter (Cytomation Inc. Fort Collins, CO). Purity of cell populations was routinely >98% for $CD4^+CD25^+$ cells and >99% for $CD4^+CD25^-$, $CD4^+CD45RB^{low}CD25^-$ and $CD4^+CD45RB^{high}CD25^-$. Purified cell populations ($2.5x10^5$ cells in 100 µl of PBS) were adoptively transferred into BALB/c SCID mice by intra-venous administration into the retro-orbital plexus.

Cell cultures and suppression assays. All cultures were set in RPMI-1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-ME, 10 mM HEPES, and 1mM sodium pyruvate (all from Life Technologies, Grand Island, NY). To measure cell proliferation and suppressor function, T cells were plated at $2.5x10^4$ cell/well in U-shape 96-well plates for 72 h together with 10^5 irradiated splenocytes as antigen-presenting cells (APC), and 0.5 µg/ml anti-CD3 mAb (clone 145.2C11; home-made). In co-cultures of T_{reg} and $CD4^+CD25^-$ cells, only the T_{reg} numbers varies according to the indicated ratios. Cultures were set in triplicates in a final volume of 200 µl. Proliferation was monitored by addition of [^{3}H] thymidine (1 µCi/well; Amersham-GE. Healthcare, Buckinghamshire, UK) for the last 6 h of culture.

Genomic and Real-time RT-PCR. *Hmox-1*$^{+/+}$, *Hmox-1*$^{+/-}$ and *Hmox-1*$^{-/-}$ mice were genotyped by PCR amplification of tail genomic DNA using two primer pairs specific to the mutated or wild type allele. Primers were as follow (5' to 3'): mutated allele TCTTGACGAGTTCTTCTGAG and ACGAAGTGACGCCATCTGT; wild type allele GGTGACAGAAGAGGCTAAG and CTGTAACTCCACCTCCAAC. For both combinations, the annealing temperature was 58ºC.

Real-time RT-PCR reactions were performed as described previously for Foxp3[9], HPRT[9] and HO-1[21]. Briefly, total RNA was extracted from 10^4 to 10^6 cells using TriPure isolation reagent (Roche Diagnostic, Mannheim, Germany), treated with DNaseI and reverse transcribed using Superscript II RT and oligo$(dT)_{12-18}$ primer (Life Technologies). Real time PCRs were performed using the QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia CA) and run on the Light

Cycler system (Roche Molecular Biochemicals, Mannheim, Germany). Foxp3, HO-1 and HPRT primer pairs were (5'-3'): Foxp3, TTC ATG CAT CAG CTC TCC ACT and AAG GTG GTG GGA GGC TGA; HO-1, TC TCA GGG GGT CAG GTC and GGA GCG GTG TCT GGG ATG; HPRT: CCA GCA AGC TTG CAA CCT TAA CCA and GTA ATG ATC GTC AAC GGG GGA C. The standard curve method, with plasmid cDNA of each gene, was applied for quantification of the amplicons. Each sample was run in triplicates. The normalized values for mRNA were calculated as the quantity of mRNA divided by HPRT mRNA levels and converted in reference to the $CD45RB^{high}CD25^{-}$ subset (=1).

Statistical analysis. Statistical significance was determined using one-way ANOVA, the Student's t test and the Mann-Whitney U test. $P<0.05$ was considered significant.

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Chapter 4: Heme Oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria.

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1. Abstract

Cerebral malaria (CM) claims more then one million lives per year. We report that heme oxygenase-1 (HO-1 encoded by *Hmox-1*) prevents the development of experimental CM (ECM). Plasmodium berghei ANKA infected BALB/c mice up-regulated HO-1 expression/activity and did not develop ECM. *Hmox-1* deletion or pharmacological inhibition of HO activity increased ECM incidence to 83% and 78%, respectively. Infected C57BL/6 mice up-regulated HO-1 to a lower extent than BALB/c mice and developed ECM (100% incidence). Pharmacological induction of HO-1 or exposure to the end product of HO-1 activity carbon monoxide (CO), reduced ECM incidence in C57BL/6 mice to 10% and 0%, respectively. HO-1 and/or CO did not affect parasitemia while preventing blood-brain barrier (BBB) disruption, brain microvasculature congestion and neuroinflammation, including CD8+ T cell brain sequestration. The mechanism underlying these effects relies on the ability of CO to bind hemoglobin, prevent its oxidation and subsequently the generation of free heme, shown hereby to trigger the pathogenesis of ECM.

2. Introduction

Plasmodium, the causative agent of malaria, causes extensive hemolysis. About 40% of the hemoglobin contained within each infected red blood cell can be released and readily oxidized[1]. This leads to the generation of free heme[2], a molecule cytotoxic to the parasite when generated within red blood cells[3] and to the host when released into the circulation[4]. *Plasmodium* has developed strategies to cope with free heme generated within red blood cells, polymerizing it into hemozoin[1]. When exposed to free heme, host cells (human or rodent) upregulate the expression of heme oxygenase-1 (HO-1), a stress-responsive enzyme that catabolizes heme into iron (Fe), biliverdin and carbon monoxide (CO)[5]. As previously shown, CO can limit the deleterious effects of inflammation[6, 7]. We hypothesized that CO might counter the pathogenesis of cerebral malaria, an inflammatory syndrome that can develop in the course of malaria infection and lead to neurological disturbances revealed clinically by abnormal behavior, impairment of consciousness, seizures and irreversible coma, ultimately leading to death[8].

C57BL/6 mice infected with *P. berghei* ANKA die within 6–12 d, due to the development of a complex neurological syndrome consisting of hemi- or paraplegia, head deviation, a tendency to roll over on stimulation, ataxia and convulsions[9]. Given its similarities to human cerebral malaria, this neurological syndrome is referred to as experimental cerebral malaria (ECM). Certain mouse strains, for example, BALB/c, are less likely to develop ECM when infected with *P. berghei* ANKA; these strains, if not treated to control parasitemia, die within 3 to 4 weeks of infection due to severe anemia and hyperparasitemia but do not exhibit neurological symptoms[9, 10].

3. Results

3.1. HO-1 prevents the development of ECM in BALB/c mice

Infection of BALB/c mice with *P. berghei* ANKA led to the upregulation of HO-1 in the brain, as assessed at the mRNA level by quantitative RT-PCR (qRT-PCR) ($P < 0.01$ comparing day 6 versus day 0; Figure 4.1A), at the protein level by western blot ($P < 0.05$, days 9–12 versus day 0; Figure 4.1B) and at a functional level by assessment of HO enzymatic activity ($P < 0.05$, day 6–12 versus day 0; Figure 4.1C). Expression of *Hmox-1* mRNA reached maximal levels 6 days after infection, decreasing thereafter until day 12, the last day analyzed (Figure 4.1A). HO-1 protein expression (Figure 4.1B) and HO activity (Figure 4.1C) reached maximal levels 9 d after infection, and the protein remained highly expressed and functional at day 12, the last day analyzed. Expression of HO-2, the constitutive form of HO, remained unchanged in the brains of infected mice, as assessed by western blot (Figure 4.1B). *Hmox-1* mRNA expression in the liver and lungs increased steadily until day 12 after infection (*data not shown*).

Upregulation of HO-1 explains the low incidence of ECM in BALB/c mice infected with *P. berghei* ANKA[11], as incidence of death with ECM-like symptoms increased from 0% in wild-type (*Hmox-1*$^{+/+}$) mice to 83.3% in HO-1 deficient (*Hmox-1*$^{-/-}$) BALB/c mice ($P < 0.001$; Figure 4.1D). Heterozygous (*Hmox-1*$^{+/-}$) and *Hmox-1*$^{+/+}$ BALB/c mice behaved similarly in that neither group developed ECM. Despite having increased ECM incidence, *Hmox-1*$^{-/-}$ mice did not have increased parasitemia, as compared to *Hmox-1*$^{+/+}$ and *Hmox-1*$^{+/-}$ mice (Figure 4.1E). This suggests that the protective effect of HO-1 does not rely on the modulation of parasitemia. We observed an apparent decrease in parasitemia in two of six *Hmox-1*$^{-/-}$ mice that did not die of ECM (Figure 4.1E); however, this decrease was not statistically significant ($P > 0.05$) and therefore its importance is difficult to ascertain.

Pharmacologic inhibition of HO enzymatic activity by zinc protoporphyrin (ZnPPIX) resulted in the death of 77.5% of infected *Hmox-1*$^{+/+}$ BALB/c mice, with symptoms consistent with the development of ECM (Figure 4.1F). ZnPPIX did not influence parasitemia (Figure 4.1G), suggesting again that the protective effect of HO-1 does not rely on the inhibition of parasitemia. Infected mice treated with cobalt protoporphyrin (CoPPIX), a protoporphyrin that induces HO-1 expression and activity, did not develop ECM (0% incidence; Figure 4.1F). CoPPIX delayed

parasitemia between days 9 and 11 after infection ($P < 0.05$ versus PBS controls), an effect lost shortly after its administration was discontinued (that is, on day 9 after infection; Figure 4.1G). As is the case with other non-iron porphyrins[12], CoPPIX might interfere with the ability of the parasite to polymerize heme into hemozoin, thereby decreasing parasitemia.

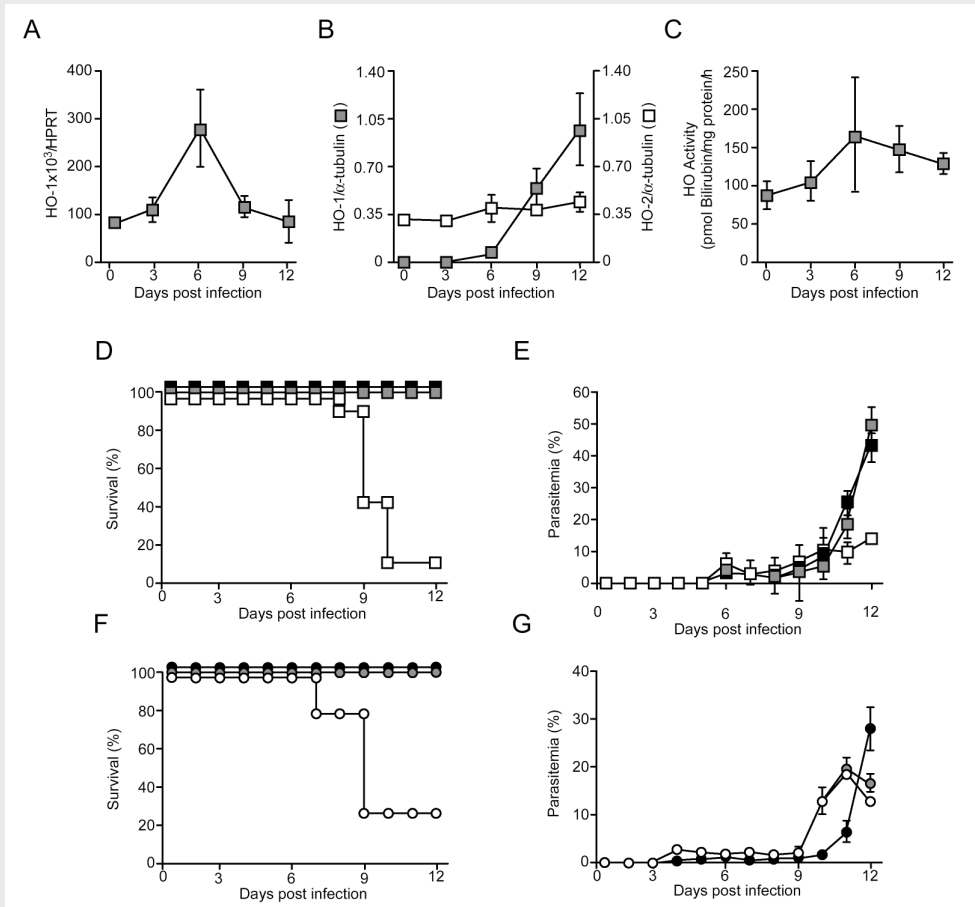


Figure 4.1- Expression of HO-1 prevents the pathogenesis of ECM. (**A**) HO-1 mRNA was quantified by qRT-PCR in the brain of *P. berghei* ANKA infected BALB/c mice and is shown as mean number (x10³) of HO-1 per HPRT mRNA molecules ± standard deviation (n=3 animals per time point). (**B**) HO-1 and HO-2 protein expression in the brain of *P. berghei* ANKA infected BALB/c mice were semi-quantified by Western blot and are shown as mean ratio of HO-1 or HO-2/a-tubulin ± standard deviation (n=4-5 animals per time point). (**C**) HO activity was quantified in the same samples as (b) and is shown as mean pmol of bilirubin per mg of brain protein per hour ± standard deviation (n=4-5 animals per time point). (**D**) Survival and (**E**) parasitemia of *P. berghei* ANKA infected *Hmox-1*$^{+/+}$ (n=20) (■), *Hmox-1*$^{+/-}$ (n= 14) (■) and *Hmox-1*$^{-/-}$ (n=12) (□) BALB/c mice. Parasitemias are shown as mean ± standard deviation. (**F, G**) Effect of HO inhibition by ZnPPIX (○) (n=10) or HO-1 induction by CoPPIX (●)(n=10) on (**F**) survival and (**G**) parasitemia of *P. berghei* ANKA infected BALB/c mice. PBS (●) treated mice were used as controls. Parasitemias are shown as mean ± standard deviation (n=5 animals per group).

3.2. HO-1 induction or exposure to CO prevent ECM onset

In contrast to BALB/c mice, C57BL/6 mice develop ECM when infected with *P. berghei* ANKA, dying within 6 to 9 d after infection[11]. ECM incidence was associated with lower levels of *Hmox-1* mRNA expression in the brains of C57BL/6 mice, as compared to BALB/c mice ($P < 0.05$; Figure 4.2A). We hypothesized that lower HO-1 expression contributes in a critical manner to the

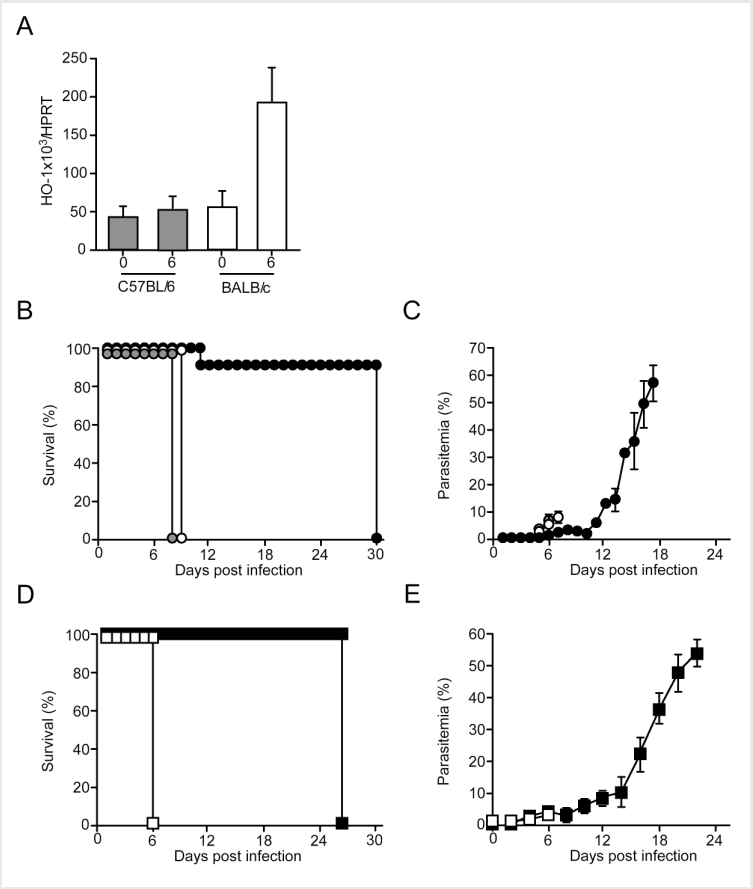


Figure 4.2- Induction of HO-1 or exposure to CO suppress ECM onset. (**A**) HO-1 mRNA was quantified by qRT-PCR in non-infected (0) *versus* P. *berghei* ANKA infected mice, day 6 post-infection (6). Number (x10^3) of HO-1 per HPRT mRNA molecules is shown as mean ± standard deviation (n=6 mice per group). (**B, C**) Effect of HO-1 induction by CoPPIX (5 mg/kg/day, by intraperitoneal injection, starting two days before infection and during 9 days thereafter) (●) on *P. berghei* ANKA infected C57BL/6 mice (**B**) survival and (**C**) parasitemia. ZnPPIX (○) or PBS (●) were used as controls (n=10 mice per group). (**D, E**) Effect of CO inhalation on *P. berghei* ANKA infected C57BL/6 mice (**D**) survival and (**E**) parasitemia. CO inhalation (250 ppm, starting at day 3 post-infection and during 24h) (■) was compared to normal atmosphere (□). Parasitemias are shown as mean ± standard deviation (n=10 animals per group).

incidence of ECM in C57BL/6 mice infected with *P. berghei* ANKA. Pharmacologic induction of HO-1 using CoPPIX reduced the incidence of ECM and death to 10%, from 100% in PBS- and ZnPPIX-treated controls (Figure 4.2B). Here too, CoPPIX led to a significant delay in parasitemia ($P < 0.05$ versus PBS-treated controls; Figure 4.2C), which, as argued above, may result from interference with parasite heme clearance[12]. As parasitemia was not treated, infected mice that did not die due

to ECM died later due to the development of hyperparasitemia (>60% infected red blood cells; Figure 4.2B, C).

CO (250 p.p.m.) given for 24 h (Figure 4.2D) or 72 h (Figure 4.3A), starting at day 3 after infection, prevented death and all ECM symptoms in C57BL/6 mice infected with *P. berghei* ANKA. None of the C57BL/6 mice exposed to CO developed ECM (Figure 4.2D). This finding is in keeping with previous observations that inhaled CO can mimic the protective effects of HO-1 in several

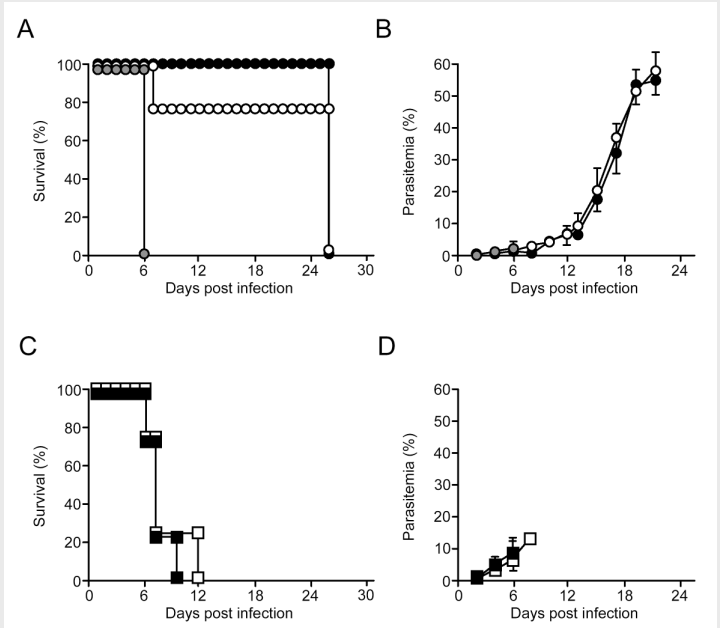


Figure 4.3- CO, but not biliverdin, suppresses the pathogenesis of ECM. C57BL/6 mice were infected with *P. berghei* ANKA. (**A**) Survival and (**B**) parasitemias are shown for air-treated controls (●) *versus* CO treated mice (n=10 per group). CO treatment was initiated either 3 days (250 ppm; total exposure time of 72h) (●) or 4 days (250 ppm; total exposure time 24h) ( ) after infection. Parasitemias are shown as mean ± standard deviation (n=10 mice per group). (**C**) Survival and (**D**) parasitemias of vehicle ( ) *versus* biliverdin-treated mice (■) (35 mg/Kg, i.p. every 8h between days 2 and 4 post-infection) (n=5 animals/group). Parasitemias are shown as mean ± standard deviation (n=4 mice/group).

experimental conditions[6, 7]. This protective effect was still observed (80% reduction in ECM incidence) when CO was administered at day 4 after infection, for 24 h (Figure 4.3A). CO administration starting at day 5 after infection was not effective, even when given before the onset of ECM clinical symptoms (*data not shown*). We observed no significant changes in parasitemia upon CO exposure (Figures 4.2E and 4.3B; $P > 0.05$), suggesting that, as with HO-1 induction, the protective effect of CO does not rely on the inhibition of parasitemia. These data suggest that at a dosage as low as 250 p.p.m., CO can be used therapeutically, that is, treatment with CO following infection can prevent ECM even when applied as late as 2 d before the expected time of death from ECM.

We assessed whether biliverdin, another end product of HO-1 activity, could mimic the protective effects of HO-1. In C57BL/6 mice infected with *P. berghei* ANKA, exogenous biliverdin did not suppress the development of ECM when administered on day 2 after infection and every 8 h thereafter until day 4 (a schedule that mimics the protective effect of HO-1 in other inflammatory conditions[13]; Figure 4.3C, D).

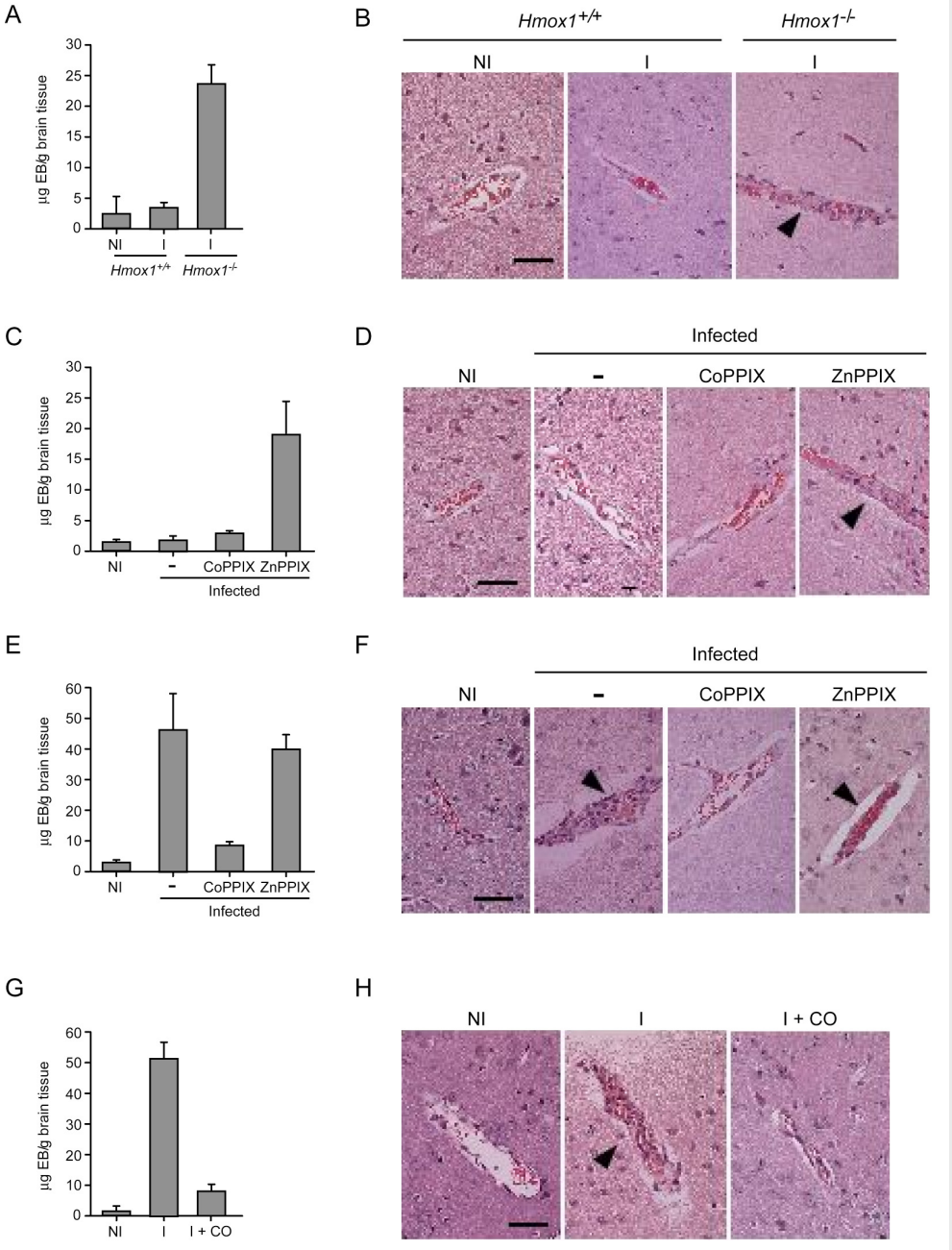
3.3. HO-1 and CO prevent BBB disruption and neuroinflammation

We confirmed ECM incidence under the different experimental conditions described above by quantifying blood-brain barrier (BBB) disruption, a hallmark of ECM as well as of cerebral malaria in humans. We also performed histological examination of brain tissue 6 to 12 d after infection.

In BALB/c mice, infection with *P. berghei* ANKA did not lead to BBB disruption (Figure 4.4A). In contrast, *Hmox-1* deletion led to BBB disruption, as revealed by a 10- to 20-fold increase in Evans blue accumulation in brain parenchyma of infected BALB/c mice, as compared to infected wild-type controls ($P < 0.0001$; Figure 4.4A). Other major pathological features associated with ECM include brain microvascular congestion with activated leukocytes and red blood cells, and brain parenchymal hemorrhages. These features were clearly detectable in *Hmox-1*$^{-/-}$ BALB/c mice infected with *P. berghei* ANKA, but not in uninfected or infected wild-type BALB/c controls (Figures 4.4B and 4.5A). In a similar manner to *Hmox-1* deletion, pharmacological inhibition of HO activity by ZnPPIX also resulted in disruption of BBB (Figure 4.4C), brain microvascular congestion (Figure 4.4D) and hemorrhage (Figure 4.5A) in infected BALB/c mice, but not in untreated or CoPPIX-treated infected controls (Figures 4.4C, D and 4.5A).

In C57BL/6 mice, infection resulted in BBB disruption: there was a 20- to 50-fold increase in Evans blue accumulation in the brain parenchyma, as compared to that in uninfected

Figure 4.4- HO-1 and CO prevent BBB disruption and brain microvascular congestion. (**A, C, E, G**) BBB disruption was assessed by Evans Blue (EB) quantification, 6 days post-infection, i.e. C57BL/6 mice or 9-12 days post-infection, i.e. BALB/c mice. Evans Blue quantification is shown as mean µg of Evans Blue (EB) per g of brain tissue ± standard deviation (n=5 animals per group). (**B, D, F, H**) Hematoxylin-Eosin staining of brain sections (5µm), analyzed at the same time as in (a, c, e and g). Images are representative of 15 mice in 3 independent experiments. Arrows indicate microvascular congestion. Bar represents 100 µm. (**A, B**) Non-infected (NI) *Hmox-1*$^{+/+}$ and *P. berghei* ANKA infected (I) *Hmox-1*$^{+/+}$ or *Hmox-1*$^{-/-}$ BALB/c mice. (**C,D**) Non-infected (NI) *versus P. berghei* ANKA infected BALB/c mice untreated (-), treated with CoPPX or ZnPPIX. (**E, F**) Non-infected (NI) *versus P. berghei* ANKA infected C57BL/6 mice untreated (-), treated with CoPPX or ZnPPIX. (**G, H**) Non-infected (NI) *versus P. berghei* ANKA infected C57BL/6 mice exposed to air (I) or CO (I+CO) (250 ppm, starting at day 3 post-infection and during 72h).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I
Hmox1+/+
Hmox1-/-
B
Hmox1+/+
Hmox1-/-
NI
I
I
C
µg EB/g brain tissue
NI
-
CoPPIX
ZnPPIX
Infected
D
Infected
NI
-
CoPPIX
ZnPPIX
E
µg EB/g brain tissue
60
50
40
30
20
10
0
NI
-
CoPPIX
ZnPPIX
Infected
F
Infected
NI
-
CoPPIX
ZnPPIX
G
µg EB/g brain tissue
NI
I
I + CO
H
NI
I
I + CO

controls (Figure 4.4E). HO-1 induction by CoPPIX reduced BBB disruption by 75.6±4.2% (mean ± s.d.; $P < 0.0001$; Figure 4.4E), and abolished brain microvascular congestion (Figure 4.4F) and hemorrhage (Figure 4.5A) associated with the onset of ECM; these effects were not seen in untreated or ZnPPIX-treated controls (Figures 4.4E, F and 4.5A).

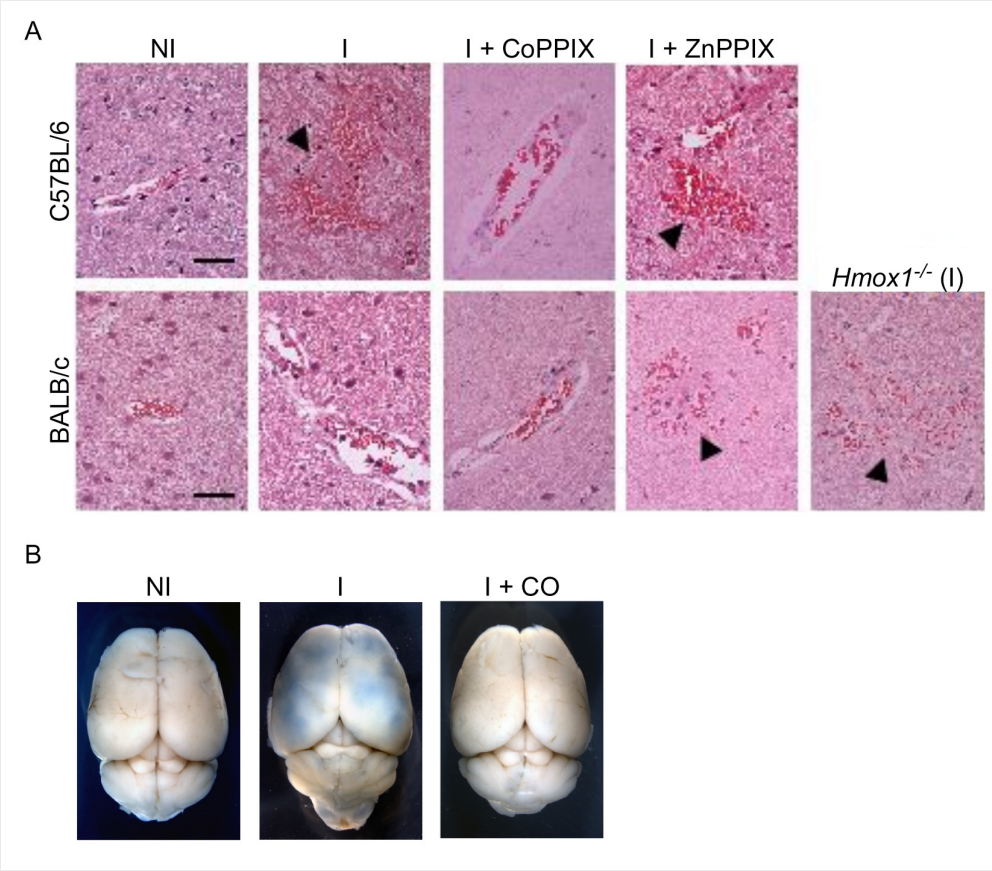

Figure 4.5- Assessment of parenchymal brain hemorrage and BBB disruption in *P. berghei* ANKA infected mice. (**A**) C57BL/6 or BALB/c mice were either not infected (NI) of infected with *P. berghei* ANKA (I). Infected mice were treated with CoPPIX (I+CoPPIX) to induce HO-1 expression/activity or with ZnPPIX (I+ZnPPIX) to suppress HO activity, as described in Methods. *Hmox-1*$^{-/-}$ BALB/c mice infected are also shown. Hematoxylin-Eosin staining of brain sections (5μm) is shown 6 days post-infection. Images are representative of 15 mice in 3 independent experiments. Arrows indicate parenchymal brain hemorrage. Bar represents 100 μm. (**B**) BBB disruption was assessed by Evans Blue staining in the brain of non-infected (NI) *versus P. berghei* ANKA infected C57BL/6 mice exposed to air (I) or CO (I+CO). Images are representative of a total of 10 mice.

Likewise, exposure to CO reduced BBB disruption by 80.2±8.1% in infected C57BL/6 mice, as compared to air-treated controls ($P < 0.0001$; Figures 4.4G and 4.5B), and abolished brain microvascular congestion (Figure 4.4H) and hemorrhage (Figure 4.5A). On the basis of previous

results[14, 15], the inhibition of BBB disruption, brain microvascular congestion and hemorrhage is likely to have a major effect in suppressing the pathogenesis of ECM.

We carried out a more detailed histological analysis of the protective effect of CO with regard to suppressing brain microvascular congestion. Development of ECM in C57BL/6 mice was associated with moderate to severe microvasculature occlusion in 58.4±13.6% of brain arterioles, capillaries or postcapillary venules. Up to 79.2±10.3% of congested microvessels presented intraluminal accumulation of both infected and uninfected red blood cells and leukocytes (91 vessels analyzed in 4 mice). Exposure to CO reduced the percentage of occluded microvessels to 24±8.3% ($P < 0.001$ *v.* air control). Only 42.6±10.8% of these microvessels contained infected red blood cells and leukocytes (250 vessels analyzed in 10 mice). In uninfected mice, 10.8±12.5% of brain microvessels had low levels of red blood cell intravascular congestion, and only 5.5±6.8% of these vessels contained leukocytes (56 vessels analyzed in 4 mice). Taken together, these data suggest that both HO-1 induction and CO exposure prevent microvascular congestion and hemorrhage in the brain, two key pathologic features of ECM and cerebral malaria[11, 16].

We asked whether CO could modulate the expression of tumor necrosis factor (TNF)[17], lymphotoxin-α (LT-α)[18] and interferon-γ (IFN-γ)[19], pro-inflammatory cytokines known to be upregulated during the pathogenesis of ECM and to contribute to its onset[16]. Expression of *Tnf* (Figure 4.6A), *Ifnγ* (Figure 4.6B) and *Ltα* (Figure 4.6C) mRNAs were significantly upregulated in the brains of C57BL/6 mice infected with *P. berghei* ANKA, as compared to uninfected controls (*Tnf*, $P < 0.001$; *Ifnγ*, $P < 0.005$; and *Lt-α*, $P < 0.05$; day 6 after infection). Exposure to CO reduced *Tnf*, *Ifn-γ* and *Lt-α* mRNA expression by 76±13% ($P < 0.002$), 98±1% ($P < 0.005$) and 68±20% ($P < 0.005$), respectively, as compared to that in infected controls (Figure 4.6A-C). Taking into consideration the critical role of pro-inflammatory cytokines in the pathogenesis of ECM[16], it is likely that the protective effect of CO is mediated at least in part through their inhibition.

Expression of intracellular adhesion molecule-1 (ICAM-1/CD54)[14] and presumably that of vascular cell adhesion molecule-1 (VCAM-1/CD106) promote the pathogenesis of ECM. *Icam1* and *Vcam1* mRNA expression was increased in the brains of C57BL/6 mice showing ECM symptoms ($P < 0.001$ and $P < 0.005$, respectively: infected *versus* uninfected mice; Figure 4.6D-E). Exposure to CO led to a 71±9% and a 41±4% decrease in ICAM-1 and VCAM-1 expression ($P < 0.001$ and

P < 0.05, respectively, infected versus infected exposed to CO), as assessed on day 6 after infection (Figure 4.6D-E).

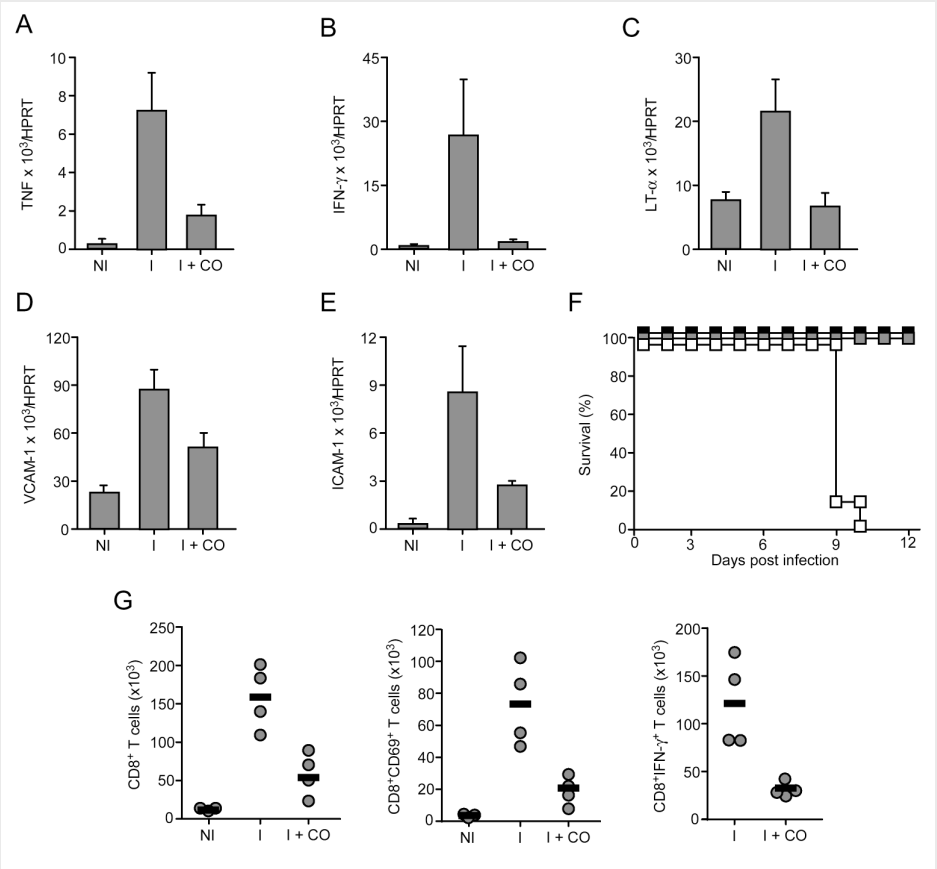

Figure 4.6- **CO inhibits neuroinflammation and CD8⁺ T cells brain sequestration.** Analyzes were performed in naïve *versus P. berghei* ANKA infected C57BL/6 mice at day 6 post-infection. CO was supplied by inhalation (250 ppm, starting at day 3 post-infection and during 72h). mRNA expression in the brain was quantified by qRT-PCR and is shown as mean number of mRNA molecules for different genes per HPRT mRNA molecules (x10³) ± standard deviation (n=5 animals per group). (**A**) TNF-α, (**B**) IFN-γ, (**C**) LT-α, (**D**) VCAM-1 and (**E**) ICAM-1 mRNA in non-infected (NI) *versus* air (I) or CO (I+CO) infected C57BL/6 mice. (**F**) Survival of *P. berghei* ANKA infected *Hmox-1*$^{-/-}$ (□), *Hmox-1*$^{-/-}$ SCID (▒) and *Hmox-1*$^{-/-}$ BALB/c mice treated with an anti-CD8 depleting antibody (■)(n=5 mice per group). (**G**) Quantification of total CD8⁺, activated CD69⁺CD8⁺ and IFN-γ⁺CD8⁺ T cells in the brain of non-infected (NI) *versus* air (I) or CO (I+CO) infected C57BL/6 mice.

In addition, we assessed whether pharmacological induction of HO-1 or exposure to CO inhibited the recruitment of CD8⁺ T cells into the brain, another critical event in the pathogenesis of ECM[20, 21]. When backcrossed into the severe combined immunodeficiency (SCID) background, *Hmox-1*$^{-/-}$ BALB/c mice infected with *P. berghei* ANKA did not develop ECM symptoms, and all

mice survived (*data not shown*). This demonstrates that HO-1 counters the deleterious effects of B or T cells that lead to ECM. Further, depletion of $CD8^+$ T cells using a CD8-specific monoclonal antibody was sufficient to prevent the onset of ECM in infected *Hmox-1*$^{-/-}$ BALB/c mice (Figure 4.6F). This result suggests that HO-1 prevents $CD8^+$ T cells from triggering ECM[20, 21]. Moreover, we assessed whether exposure to CO (Figure 4.6G) or the induction of HO-1 by CoPPIX (*data not shown*) could suppress $CD8^+$ T-cell sequestration in the brain. In infected C57BL/6 mice, CO decreased the total number of $CD8^+$ T cells in the brain (including activated $CD8^+CD69^+$ and IFN-γ–secreting $CD8^+$ T cells) by 65–75%, as compared to air-treated controls ($P < 0.05$; Figure 4.6G). CO also inhibited monocyte/macrophage ($CD45RB^{high}CD11b^+$) and PMN ($CD45RB^{high}GR1^+$) recruitment into the brain (*data not shown*). Induction of HO-1 by CoPPIX yielded similar results to CO inhalation, whereas ZnPPIX had no effect (*data not shown*). These results suggest that CO suppresses the sequestration of $CD8^+$ T cells in brains of

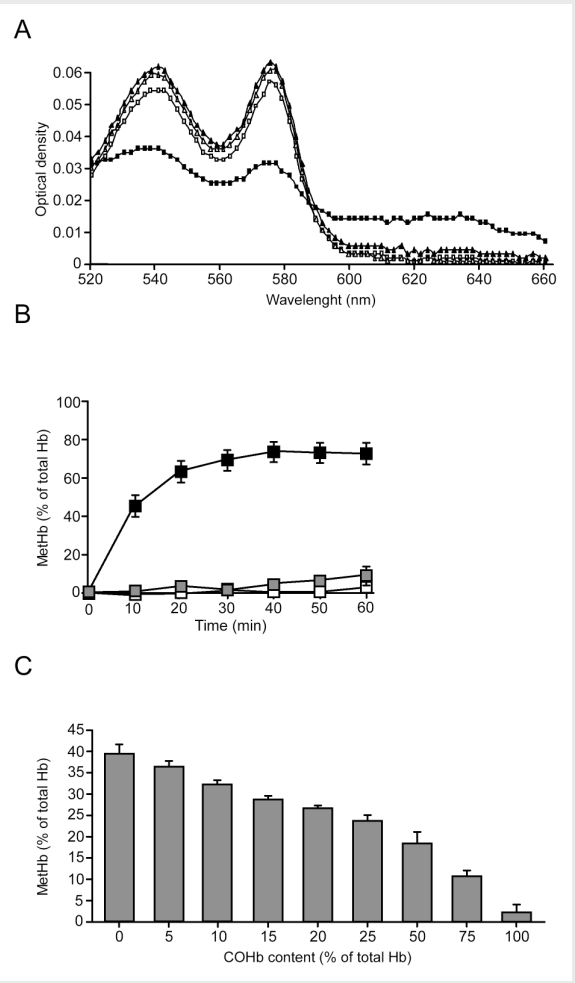

Figure 4.7- CO prevents Hb oxidation. (**A**) Hb (200 μM) was exposed to CO (100% bubbled for 10 seconds at a flow rate of 10 mL/min) and subjected to oxidation by H2O2 (2 mM, 1h, 37 oC). Wavelength scans illustrate untreated Hb ( ), Hb + H2O2 (◇), Hb + CO (◇), Hb + CO + H2O2 (■). (**B**) Hb was exposed to CO as in (A) and subjected to oxidation by activated human polymorphonuclear (PMN) cells (4x107/ml, 1h). Methemoglobin (MetHb) formation is shown as mean percentage of total Hb ± standard deviation in samples containing untreated Hb ( ), Hb + PMN cells (■) and Hb + CO + PMN cells (■) (n=3 independent experiments). (**C**) CO inhibits H2O2-mediated methemoglobin (MetHb) formation in a dose dependent manner. Hb (200 mM) containing different carboxyhemoglobin (COHb) percentages was exposed to H2O2 (2 mM) for 1 hour at 37 oC. MetHb formation was assessed spectrophotometrically. Results shown are the mean percentage of MetHb ± standard deviation (n=3 independent experiments).

mice infected with *P. berghei* ANKA, an effect that should contribute to the suppression of ECM onset[20, 21].

3.4. CO inhibits heme release from oxidized hemoglobin

Malaria is associated with a severe hemolysis that generates extracellular ferrous (Fe^{2+}) hemoglobin, which in the presence of reactive oxygen species (ROS) is readily oxidized into methemoglobin (MetHb) (Fe^{3+}). We asked whether CO could arrest this process. Generation of carboxyHb (COHb; 100%) by exposure of purified ferrous (Fe^{2+}) hemoglobin to CO *in vitro* suppressed MetHb formation driven by either H_2O_2 or activated polymorphonuclear (PMN) cells (>85% inhibition versus that in air-treated controls, $P < 0.0001$; Figure 4.7A, B). These effects were dose dependent, as increasing the percentage of COHb led to increased inhibition of MetHb formation (Figure 4.7C), and were not observed when PMN were exposed to CO instead of Fe^{2+}

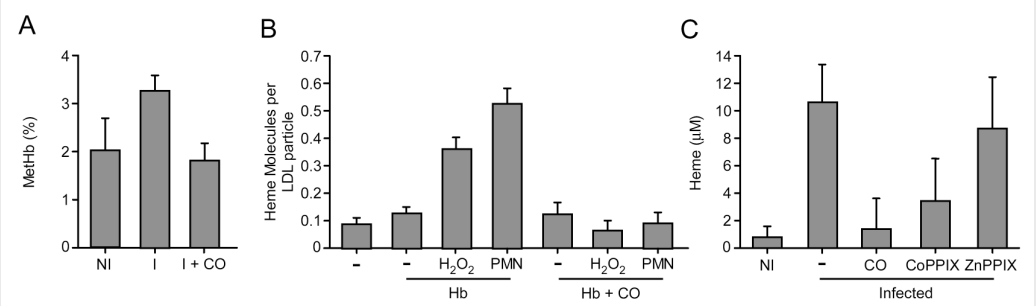

Figure 4.8 - **HO-1 and CO inhibit free heme release from oxidized Hb.** (**A**) Percentage of methemoglobin (MetHb) in whole blood of non-infected (NI) and *P. berghei* ANKA infected C57BL/6 mice exposed to air (I) or to CO (I+CO) (250 ppm, starting at day 3 post-infection and during 72h). Measurements were performed at day 6 post-infection. Results are shown as mean percentage of MetHb ± standard deviation (n=5 mice per group). (**B**) Hb (200 µM) was exposed to air or to CO (100% bubbled at a flow rate of 10 mL/min for 10 seconds) to generate carboxyHb (COHb) and then subjected to oxidation by H_2O_2 (2 mM, 1h, 37 °C) or by activated polymorphonuclear (PMN) cells ($4x10^7$/ml, 1h). Free heme captured by low density lipoprotein (LDL) is shown as mean number of heme per LDL molecules ± standard deviation (n=3 independent experiments). (-) represents the background of the technique. (**C**) Free heme concentration in protein-free plasma of non-infected (NI) *versus P. berghei* ANKA infected C57BL/6 mice exposed to air (-) or CO (250 ppm, starting at day 3 post infection and during 72 hours) and analyzed at day 6 post-infection. Results shown are mean plasma heme concentration ± standard deviation (n=4-7 mice per group).

hemoglobin (*data not shown*). These results suggested that CO prevents hemoglobin oxidation through a mechanism that relies on its binding to Fe^{2+} hemoglobin. To ascertain whether similar effects occur *in vivo*, we measured MetHb concentration in whole blood from infected C57BL/6 mice that had or had not been exposed to CO. MetHb concentration increased significantly in infected versus uninfected mice ($P < 0.05$; Figure 4.8A). Exposure of the infected mice to CO (250

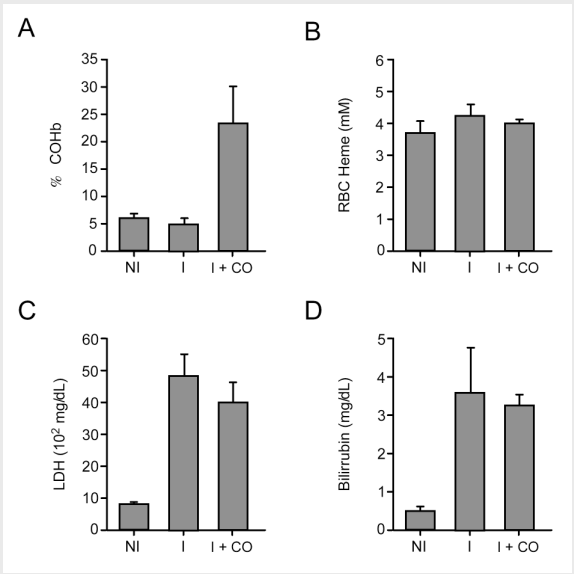

Figure 4.9- CO does not inhibit red blood cell lysis. (**A**) Percentage of carboxyhemoglobin (COHb) in whole blood from non-infected (NI) and *P. berghei* ANKA infected C57BL/6 mice exposed to air (I) or CO (I+CO) (250 ppm, starting at day 3 post-infection and during 24h thereafter). Measurements were carried immediately after CO exposure. Values shown are the mean percentage of COHb ± standard deviation (n=5 animals/group). (**B**) Heme concentration in circulating red blood cells (RBC) of non infected (NI) and *P. berghei* ANKA-infected C57BL/6 mice exposed to air (I) or CO (I+CO) (250 ppm, starting at day 3 post-infection and during 72 h thereafter). Measurements were carried out at day 6 post infection. Results shown are the mean heme concentration ± standard deviation (n=4-7 animals/group). (**C**) Lactate dehydrogenase (LDH) and (**D**) direct bilirubin concentrations in the serum of non-infected (NI) and *P.berghei* ANKA infected C57BL/6 mice exposed to air (I) or CO (I+CO) as in (b). Measurements were carried out at day 6 post infection. Results are shown as mean concentration ± standard deviation (n=5mice per group).

p.p.m.; 23±6% COHb) decreased MetHb concentration to basal levels (that is, to levels observed in uninfected mice; Figure 4.8A).

MetHb is highly unstable, releasing free heme[22]. The ability of H_2O_2 or activated PMN cells to release heme from Fe^{2+} hemoglobin was suppressed when hemoglobin was exposed to CO before oxidation (>95% inhibition *versus* air-treated controls, $P < 0.0001$; Figure 4.8B). These data indicate that once bound to Fe^{2+} hemoglobin, CO suppresses not only its oxidation but also the subsequent generation of free heme. To assess whether a similar mechanism occurs *in vivo*, we measured the concentration of extracellular (non-protein-bound) heme in the circulation of infected C57BL/6 mice (Figure 4.8C). Free heme concentration increased significantly upon infection ($P < 0.001$; Figure 4.8C). Both exposure to CO and the induction of HO-1 using CoPPIX reduced plasma free heme concentration by 88±22% and 69± 31%, respectively, as compared to that in untreated infected controls ($P < 0.01$; Figure 4.8C). With ZnPPIX treatment, plasma free heme concentration was not different than that in untreated infected controls (Figure 4.8C).

CO did not modulate red blood cell counts (*data not shown*), heme red blood cell content (Figure 4.9B), lactate dehydrogenase (LDH) levels (Figure 4.9C) or bilirubin concentration (Figure 4.9D) in the circulation of infected C57BL/6 mice. These results suggest that CO inhibits the

accumulation of free heme in the circulation of these mice by a process that does not involve the inhibition of red blood cell lysis.

3.5. Free heme triggers ECM

We asked whether the accumulation of free heme in the circulation of infected C57BL/6 mice was involved in the pathogenesis of ECM. Heme administration 3 d after infection was sufficient to reverse the protective effect of CO, causing death in 100% of infected mice, with symptoms consistent with ECM, compared to 0% of vehicle-treated mice (Figure 4.10A). Heme had no deleterious effects when administered to uninfected C57BL/6 mice at the same dose and on the same schedule (*data not shown*).

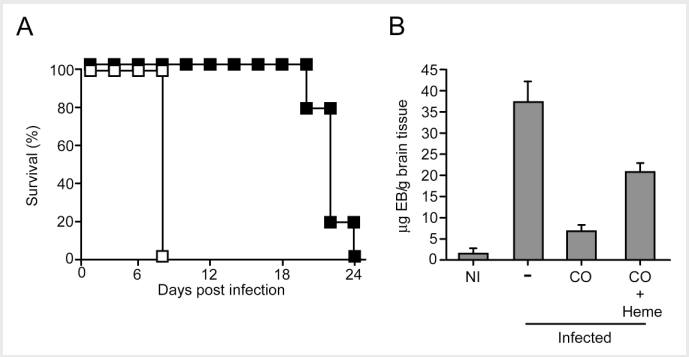

Figure 4.10- Free heme administration counters the protective effect of CO. (**A**) Survival of *P. berghei* ANKA infected C57BL/6 mice exposed to CO (250 ppm, starting at day 3 post infection and during 72 hours) and receiving heme (□; i.p., 60mg/kg, 120 and 132h post-infection) or vehicle (■). (**B**) BBB disruption in non-infected (NI) *versus P. berghei* ANKA infected C57BL/6 mice exposed to air (-), CO (250 ppm, day 3 post-infection,72h) or CO plus heme (i.p.; 40mg/kg; starting 3 days post infection and every 12 hours thereafter). Evans Blue (EB) quantification is shown as mean µg of Evans Blue per g of brain tissue ± standard deviation (n=4-5 mice per group).

Heme administration reverted the protective effect of CO and triggered BBB disruption, a key pathological feature of ECM and cerebral malaria (Figure 4.10B). These data reveal not only that accumulation of free heme in the circulation of infected mice is a central component in the pathogenesis of ECM, but also that CO suppresses ECM by inhibiting this process, presumably by blocking hemoglobin oxidation.

We tested whether the ability of different *Plasmodium* strains to trigger ECM was functionally linked to the amount of free heme that accumulates in the host circulation. Plasma free heme concentration was reduced by 44±19% ($P < 0.001$) in C57BL/6 mice infected with *P. berghei* NK65 versus *P. berghei* ANKA (Figure 4.11A). The former group did not succumb, whereas the latter succumbed 6–7 d after infection, with symptoms consistent with ECM (Figure 4.11B). After this time period, free heme concentrations remained constant in mice infected with *P. berghei*

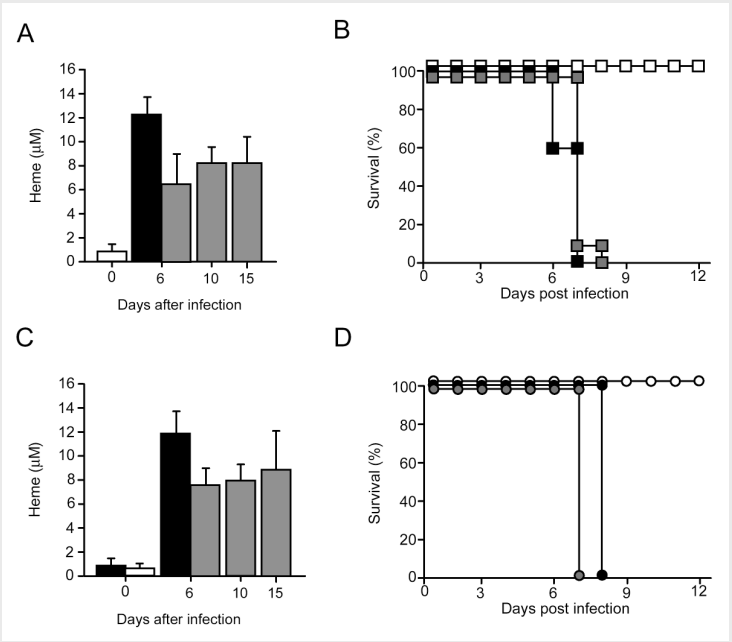

Figure 4.11- Correlation between circulating free-heme and susceptibility to ECM. C57BL/6 or BALB/c mice were not infected or infected with *P. berghei* ANKA or *P. berghei* NK65. Circulating free heme concentration (A and C) and survival (B and D) were assessed. (**A**) Heme concentration in protein free plasma of non-infected (white histogram)(n=7), *P. berghei* ANKA (black histogram) or NK65 (grey histogram) infected C57BL/6 mice. Measurements were carried out at days 6 (n=30 and 16 for *P. berghei* ANKA and NK65, respectively), 10 (n=10) and 15 (n=10) post-infection. Results are shown as mean heme concentration ± standard deviation. (**B**) Survival of C57BL/6 mice infected with *P. berghei* ANKA (■)(n=10), *P. berghei* NK65 (□)(n=10) or *P. berghei* NK65 plus hemin (60 mg/kg: i.p.; day 6 after infection) (▩) (n=12). (**C**) Heme concentration in protein free plasma of non-infected C57BL/6 mice (black histogram; day 0)(n=7), non-infected BALB/c mice (white histogram; day 0)(n=6), *P. berghei* ANKA C57BL/6 mice (black histogram; day 6) (n=30) or *P. berghei* ANKA infected BALB/c mice (white histograms; day 6) (n=15), 10 (n=10) and 15 (n=12) post-infection. Results are shown as mean heme concentration ± standard deviation. (**D**) Survival of *P. berghei* ANKA infected C57BL/6 mice (●)(n=10), BALB/c mice (○)(n=5) or BALB/c mice receiving hemin (60 mg/kg: i.p; day 6 post infection) (◍) (n=6).

ANKA NK65: at days 10 and 15 after infection, concentratios were 33±11% and 34±19%, respectively, of those in mice infected with *P. berghei* ANKA (P < 0.001; Figure 4.11A). Heme administration to C57BL/6 mice infected with *P. berghei* ANKA NK65 (60 mg per kg body weight; at 6 d after infection) led to death in 100% of mice, with symptoms consistent with ECM (Figure 4.11B). Heme administration to uninfected C57BL/6 mice at the same dose and on the same schedule was not lethal (*data not shown*).

We then asked whether the absence of ECM in *P. berghei* ANKA–infected BALB/c mice compared to its occurrence in *P. berghei* ANKA–infected C57BL/6 mice was also functionally linked to the accumulation of circulating free heme. Plasma free heme concentration was reduced by 36±9% (P < 0.001) in BALB/c versus C57BL/6 mice (Figure 4.11C). At the time of development of ECM symptoms, plasma free heme concentration in BALB/c mice was reduced by 36±9% and 28±32% at days 10 and 15 after infection, respectively, from the levels in infected C57BL/6 mice (P < 0.001; Figure 4.11C). Heme administration to BALB/c mice infected with *P. berghei* ANKA (60

mg/kg; i.p.; 6 d after infection) led to death in 100% of the mice, with symptoms consistent with ECM (Figure 4.11D). Heme administration to uninfected BALB/c mice was not lethal (*data not shown*). Taken together, these data support the notion that accumulation of free heme in the circulation of a malaria-infected host plays a critical role in dictating the host's susceptibility to ECM.

We hypothesized that free heme might affect BBB tight-junction function in a manner that would promote BBB disruption. We tested this hypothesis using a well-established *in vitro* BBB model, in which confluent bovine brain microvascular endothelial cells (bMVEC-B cells) form functional tight junctions, revealed by the exclusion of a fluorescent 10-kDa dextran tracer in a transwell assay (Figure 4.12). Tight-junction functional integrity was not disrupted when bMVEC-B cells were exposed to either H_2O_2 or heme alone. However, when bMVEC-B cells were pre-exposed to heme, further addition of H_2O_2 caused loss of functional tight-junction integrity, as revealed by a 2–5 fold increase in fluorescent dextran tracer permeability. The effect of heme was dose dependent, with higher concentrations leading to increased permeability. We obtained similar results using Madin-Darby canine kidney (MDCK) cells (*data not shown*), which also form tight junctions *in vitro*. These data suggest that in the presence of a ROS (that is, H_2O_2), heme can disrupt BBB tight-junction function, which could explain the BBB disruption associated with the pathogenesis of ECM. By blocking the generation of free heme, CO may act to prevent BBB disruption.

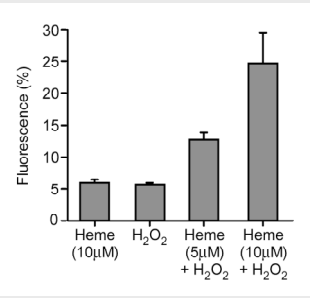

Figure 4.12- Free heme promotes BBB disruption. Permeability of confluent bMVEC-B cells to 10-kDa fluorescent dextran was quantified in a in a transwell chamber, after exposure to heme, H_2O_2 (100 μM) or heme (1h) + H_2O_2 (100 μM). Results are shown as mean percentage of fluorescent tracer in the lower chamber ± standard deviation (n=3).

4. Discussion

Understanding the pathogenesis of malaria infection is crucial for the development of more efficient clinical interventions. Sequestration of infected red blood cells and leukocytes (that is, $CD8^+$ T cells in the brain[20-22]), and the inflammatory response triggered by malaria infection are thought to be the two key events in the pathogenesis of cerebral malaria[8]. Our present data show that HO-1 expression and enzymatic activity in the host counter the pathogenesis of ECM. Moreover, we have demonstrated that the administration of exogenous CO via inhalation can be used therapeutically to suppress the pathogenesis of ECM. The protective effects of CO administration were associated with inhibition of BBB disruption, brain microvascular congestion and hemorrhage, as well as with suppression of neuroinflammation, including inhibition of adhesion molecule expression in the brain microvasculature and the suppression of activated $CD8^+$ T cell sequestration in the brain.

The ability of CO to suppress the accumulation of free heme in the circulation of the infected host seems critical for its protective effects. Malaria infection is associated with severe hemolysis and therefore with the oxidation of cell-free hemoglobin[2], leading to the release of free heme and its accumulation in plasma. We have shown that circulating free heme is a central effector in the pathogenesis of ECM, promoting BBB disruption by means of a mechanism targeting the tight junctions that maintain BBB functional integrity. Binding of CO to Fe^{2+} in the heme groups of ferrous hemoglobin prevents hemoglobin oxidation, heme release from oxidized hemoglobin, accumulation of free heme in the circulation, BBB disruption and, consequently, the development of ECM.

Other researchers have shown that, as in other hemolytic diseases (for example, sickle cell anemia[23]), scavenging of nitric oxide (NO) by cell-free hemoglobin generated during malaria infection promotes the onset of ECM[24]. It is possible therefore that CO and NO might interact functionally to suppress the pathogenesis of ECM. Once bound to cell-free hemoglobin, CO may limit NO scavenging, an effect that would increase NO bioavailability and thus suppress the pathogenesis of ECM[24]. According to this notion, the protective effect of CO might be 'NO dependent'. On the other hand, the protective effect of NO might itself be 'CO dependent', as NO is a potent inducer of HO expression[25]. NO might act in a protective manner through the induction of

HO-1 and the subsequent generation of CO, shown here to prevent the onset of ECM. These hypotheses remain to be tested experimentally.

The proposed mechanism for the protective action of CO (Figure 4.13) may have important implications for understanding the pathogenesis of cerebral malaria. Expression of HO-1 occurs during malaria infection in humans[26, 27], suggesting that a similar protective mechanism may operate to suppress the development of cerebral malaria. Although ECM may not fully reflect human cerebral malaria, it allows for the identification of genes that control both of these pathologic processes, as shown for the involvement of *TNF*[17, 28-30] and *ICAM1* (also known as CD54)[31, 32] in ECM and cerebral malaria[33]. Thus, although our data do not allow to conclude unequivocally that *HMOX-1* is a gene that determines susceptibility to cerebral malaria, it is likely that variations in HO-1 expression, known to occur in human populations as a result of a (GT)n microsatellite polymorphism in the promoter region of the human *HMOX-1* gene[34], can dictate susceptibility to cerebral malaria.

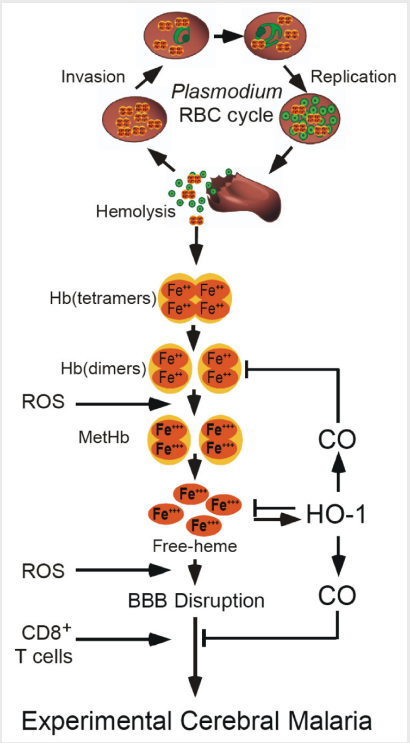

Figure 4.13- Mechanism underlying the protective action of HO-1 and CO. Hemolysis is an inherent process associated with the cycles of red blood cell (RBC) invasion/ replication/ egress by *Plasmodium.* This process leads to the release of Hb into the circulation. Cell-free Hb dimerizes spontaneously and in the presence of ROS is readily oxidized into MetHb. This latter process of Hb oxidation will lead to the prompt release of heme. In the presence of ROS cell-free heme will promote BBB disruption, which in the presence of activated $CD8^+$ T cells will trigger the onset of ECM. Both heme and ROS can up-regulate the expression of HO-1, a stress responsive enzyme that generates CO via heme degradation. CO can bind cell-free Hb dimers, blocking their oxidation and thus the generation of circulating free heme. In addition, CO also prevents the recruitment of $CD8^+$ T cells in the brain microvasculature. These effects may act in a concerted way to afford potent protection against the development of ECM.

malaria infection in mice, which occurs before the blood stage. Moreover, we found that the liver stage of infection was reduced by 70–80% in *Hmox1*$^{-/-}$ mice (S. Epiphanio *et al*., *unpublished data*). These findings suggest that a successful host-*Plasmodium* interaction leading to long-lasting infection in a viable host is quite complex and is dependent on the regulated expression of HO-1 in different tissues at different stages of the *Plasmodium* life cycle.

This study reveals that the pathogenesis of cerebral malaria might be controlled through the expression of a so-called 'protective gene' in the host[36]. We have shown this for HO-1 in the context of ECM, but we do not exclude the possibility that other 'protective genes'[37] might act in a similar manner to prevent the onset of cerebral malaria or other forms of severe acute malaria in humans. These findings provide new insights into the pathogenesis of ECM and may lead to new therapeutic approaches to suppress the pathogenesis of human cerebral malaria based on the modulation of HO-1 expression or the administration of CO.

5. Methods

Mice. C57BL/6 and BALB/c mice were bred and housed in the pathogen-free facilities of the Instituto de Gulbenkian de Ciência. All protocols were approved by the Animal Care Committee of the Instituto Gulbenkian de Ciência. We generated *Hmox-1*$^{-/-}$ mice by mating *Hmox-1*$^{+/-}$ mice (backcrossed for ten generations into the BALB/c background), as previously described[38]. We backcrossed BALB/c *Hmox-1*$^{+/-}$ mice into the severe combined immunodeficient (SCID) background (Jackson Laboratory). We isolated genomic DNA from the tail and determined the *Hmox* genotype by PCR 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'. We repeated each PCR reaction at least three times before experiments on the mice were performed and once more afterward. As controls, we used littermate *Hmox-1*$^{+/-}$ and *Hmox-1*$^{+/+}$ mice.

Parasites, infection and disease assessment. In all experiments, we used red blood cells infected with green fluorescent protein (GFP)-transgenic *P. berghei* ANKA[39] or *P. berghei* NK65 to infect C57BL/6 or BALB/c mice. Mice were infected by intraperitoneal (i.p.) inoculation of 10^5 infected red blood cells, except in experiments using *Hmox-1*-deficient mice, in which 10^4 infected red blood cells were used. Infected mice were monitored twice daily for clinical symptoms of ECM including hemi- or paraplegia, head deviation, tendency to roll over on stimulation, ataxia and convulsions. We determined parasitemia by flow cytometry for mice infected with GFP-transgenic *P. berghei* ANKA and by Giemsa staining followed by microscopic counting for mice infected with *P. berghei* NK65. These results are expressed as percentage of infected red blood cells, as previously described[39].

Protoporphyrins. Iron protoporphyrin-IX (FePPIX, hemin), zinc protoporphyrin-IX (ZnPPIX), cobalt protoporphyrin-IX (CoPPIX) and biliverdin hydrochloride (BV) (Frontier Scientific Inc.), were dissolved in 0.2 M NaOH, neutralized (to pH 7.2) with 1 M HCl, and adjusted to concentrations of 1 mg/ml (ZnPPIX, CoPPIX) or 3.8 mg/ml (BV) with distilled water. Aliquots were stored at -80 °C and protected from light until used. For BBB studies, we treated mice with FePPIX (40 mg/kg body

weight) every 12 h, starting on day 3 after infection. For survival studies, we administered FePPIX (60 mg/kg body weight) every 12 h, starting on day 5 after infection. CoPPIX (5 mg/kg body weight per d) and ZnPPIX (5 mg/kg/d) were administered i.p. and were started 2 d before infection and continued for 9 d thereafter. We administered biliverdin every 8 h (35 mg/kg i.p.) between days 2 and 4 after infection.

CO exposure. Mice were placed in a gastight 60-liter capacity chamber and exposed to CO for the times indicated in the figure legends, as described elsewhere[6, 40]. Briefly, 1% CO (Aga Linde) was mixed with air in a stainless steel cylinder to obtain a final concentration of 250 p.p.m. CO was provided continuously at a flow rate of ~12 liter/min, starting on day 3 after infection and continuing for 72 h, unless otherwise stated. We monitored CO concentration using a CO analyzer (Interscan Corporation). Controls were maintained in a similar chamber without CO. We measured levels of COHb using a portable CO-oximeter (AVOXImeter 4000, Avox Systems).

BBB permeability. We injected mice intravenously (i.v.) with 0.2 ml of 1–2% Evans blue (Sigma) when clinical symptoms of ECM were observed (that is, head deviation, convulsions, ataxia and paraplegia). Mice were killed 1 h thereafter, and brains were weighed and placed in formamide (2 ml, 37 °C, 48 h; Merck) to extract Evans blue dye from the tissue, essentially as previously described[41]. Absorbance was measured at λ= 620 nm (Bio Rad SmartSpec 3000). We calculated Evans blue concentration using a standard curve. Data are expressed as μg of Evans blue per g of brain tissue.

CD8-specific antibody depletion. *Hmox-1*$^{-/-}$ BALB/c mice infected with *P. berghei* ANKA received 0.8 mg of CD8-specific monoclonal antibody (clone 2.43, ATCC) on days 5 and 8 after infection, a protocol leading to CD8^{+} T-cell depletion and suppression of ECM[20].

Heme quantification *in vivo*. Blood was drawn into heparinized tubes by heart puncture and centrifuged (5 min, 4 °C, 1,100*g*). We collected red blood cells, centrifuged the plasma (5 min, 4 °C, 1,100*g*) to remove contaminating red blood cells and passed it through a Microcon YM-3

column (Millipore)(60 min at 14 °C, 21,000*g*) to remove proteins (MW > 3 kDa). We quantified heme (that is, free heme) in protein-depleted plasma using a chromogenic assay according to the manufacturer's instructions (QuantiChrom heme assay kit, Bioassay Systems). We washed red blood cells in a choline washing solution (160 mM choline chloride, 10 mM glucose, 10 mM Tris.MOPS, pH 7.4); the cells were incubated in washing solution for 5 min at 4 °C, then centrifuged at 1,100*g*, and this was repeated 4 times. We lysed the cells in H_2O (1/100 vol) and measured heme concentration using the same chromogenic assay as above.

Statistical analysis. For samples in which $n > 5$, statistical analyses were performed using the unpaired Student's *t*-test or analysis of variance (ANOVA) parametric tests. Normal distributions were confirmed using the Kolmogorov-Smirnov test. For samples in which $n < 5$, statistical analyses were performed using Kruskall-Wallis or Wilcoxon nonparametric tests. The log-rank test was used for all experiments in which survival was assessed as an end point. $P < 0.05$ was considered significant; $P < 0.001$ was considered highly significant.

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Chapter 5: General Discussion

Biology is, in its broadest sense, a comprehensive attempt aimed at solving a mosaic with a finite, but extremely large, number of pieces[307]. Undoubtly, inflammation and immunity constitute pieces of such puzzle that have for a long time mesmerized and instigated many questions in investigators. As a central process by which the immune system counters damage arising from microbial infection as well as chemical or physical insults, inflammation is, in the majority of cases, a salutary self-limiting and self-resolving response. Inflammatory reactions are in most cases associated with tissue regeneration and return to homeostasis. However, failure in resolving an inflammatory reaction can result in the development of chronic inflammatory diseases. In the last decades, it has become apparent that unfettered inflammation is not only causally related to the pathogenesis of inflammatory diseases, but also contributes in a critical way for the pathological outcome of molecular and infectious diseases. Given the impact of inflammation in such a broad number of pathologic conditions (*see Chapter 1, Table 1.2*), a better understanding of the cellular and molecular mechanisms controlling inflammatory reactions is essential to devise therapeutic strategies aimed at overcoming these diseases. Several mechanisms involved in the resolution of inflammation have been identified (*see Chapter 1, section 1.1.3.*). One of such mechanisms relies on the expression of protective genes, which act not only by counteracting the cytotoxic effects of inflammation but also by limiting the extent of the inflammatory reaction itself. A growing body of evidence suggests that HO-1 acts in such a manner (*see Chapter 1, section 2.*).

The role of HO-1 in the pathogenesis of autoimmune neuroinflammation was assessed using EAE (*see Chapter 2*), an animal model that recapitulates most of the immunopathologic events associated with the progression of this disease in humans (*see Chapter 1, section 3.1.2.*). Endogenous expression of HO-1 has been previously described in several pathologic conditions that target the CNS, including brain ischemia[308], trauma[309] and intracerebral hemorrhage[310]. Likewise, expression of HO-1 was also known to be induced during the course of autoimmune neuroinflammation both in rodents and humans[311] and to be mainly restricted to infiltrating Mø although activated microglia and astrocytes also express high levels of HO-1[312, 313]. Analysis of the kinetics of HO-1 expression in rats undergoing EAE revealed that its expression is induced during lesion formation and that elevated levels of expression are maintained during EAE resolution even after neurological symptoms have cessed[312]. The apparent overlap between HO-1 expression and

disease resolution led us to hypothesize that HO-1 might play an important role in limiting the extent of neuroinflammation associated with the clinical course of EAE and MS.

To address this hypothesis unequivocally we made use of genetically engineered mice in which the 3rd exon of the *Hmox-1* gene was disrupted by homologous recombination, suppressing HO-1 expression[314]. Active immunization of *Hmox-1*$^{-/-}$ mice resulted in exacerbated demyelination and paralysis leading to increased mortality, as compared to *Hmox-1*$^{+/+}$ mice (*see Chapter 2, section 3.1.*). This finding underscore the protection afforded by endogenous HO-1 expression during the establishment and progression of autoimmune neuroinflammation. Although our work suggests that the mechanism by which HO-1 affords protection against EAE involves the modulation of activation and effector function of autoreactive T_H cell clones, presumably via modulation of DC activity (*see Chapter 2 and below*), it is possible that HO-1 might also afford cytoprotection to those cells in the CNS directly targeted by this autoimmune response, i.e. ODCs, and/or neurons. In keeping with this notion, activation of stress-induced mechanisms in ODCs and neurons have been proposed to prevent tissue damage and clinical manifestations associated with CNS pathology. Such is the case of NF-E2-related factor (Nrf)2, a transcription factor involved in cellular defence against oxidants and toxic chemicals and critically involved in the induction of HO-1 expression. Mice deficient in *Nrf2*$^{-/-}$ exhibit vacuolar leukoencephalopathy characterized by myelin destruction due to localized oxidative damage, implicating Nrf2, and thus its target genes, e.g. HO-1, in the maintenance of the myelin sheath in the CNS[315]. More importantly, both Nrf2 and HO-1 expression can protect neurons from glutamate-mediated excitotoxicity[316, 317], an effector mechanism involved in the pathogenesis of autoimmune neuroinflammation (*see Chapter 1, section 3.1.8.1.*). It is likely, therefore, that the signalling pathway leading to Nrf2 activation and concomitant expression of its target genes, including HO-1, might protect ODCs from glutamate-mediated oxidative damage during the course of EAE and presumably that of MS as well.

While cytoprotection *per se* is likely to ameliorate the clinical outcome of autoimmune neuroinflammation by sustaining tissue function, such an effect may act in additional ways to afford protection against disease development. By limiting ODC and neuronal injury, Nrf2/HO-1 might reduce the release of neuroantigens and/or putative DAMPs. Presumably, such an effect should have a significant impact on the activation of APCs, i.e. DCs, and thus on the activation and/or

effector function of autoreactive T_H cells as well as in that of CNS-infiltrating Mø thus limiting ongoing neuroinflammation and epitope spreading (*see Chapter 1, section 3.1.4.*).

Further from establishing that endogenous HO-1 expression controls the pathologic outcome of EAE, we also found that pharmacological induction of HO-1 expression can act therapeutically to reverse ongoing autoimmune neuroinflammation (*see Chapter 2, section 3.1*). It is well established that substitution of Fe in the protoporphyrin IX core by other metals results in compounds that can function as inducers or inhibitors of HO-1 activity. Metalloporphyrins containing zinc (Zn) or tin (Sn), i.e. ZnPPIX and SnPPIX respectively, compete with heme for the binding to the catalytic site in the HO-1 protein resulting in inhibition of HO activity[318]. On the other hand, cobalt (Co) protoporphyrin IX (CoPPIX) induces HO-1 expression[319] by selectively modulating the protein levels of the Nrf2/Bach1 axis[320]. The potent inducibility of HO-1 expression afforded by CoPPIX coupled with its inability to compete for the catalytic site of the enzyme[321] results in enhanced HO-1 activity. Pharmacological induction of HO-1 by CoPPIX administration after the onset of clinical disease reverts paralysis associated with the progression of EAE. This therapeutic effect is independent from the genetic background of the mice or the encephalitogenic peptide used to actively induce EAE (*see Chapter 2, section 3.1*). This finding suggests that HO activity is involved in its protective effect, which led to the hypothesis that CO, a cytoprotective and anti-inflammatory end-product of heme catabolism by HO-1 (*see Chapter 1, section 2.3.1.*), may mediate the protection afforded by HO-1. We tested this hypothesis and found that indeed, CO inhalation affords similar protective effects to those of HO-1 induction (*see Chapter 2, section 3.1.*). However, the effects of CO where more restricted than those observed upon induction of HO-1 expression (*see Chapter 2, section 3.4.*) suggesting that other end-products of heme degradation by HO-1, including BV, might contribute to the protective effect of HO-1 during EAE progression. However, our present data suggest that BV does not suppress myelin-reactive T_H cell proliferation nor does it suppress EAE progression (*see Chapter 2, section 4.*). Notwithstanding, BR, generated from the reduction of BV by BVR (*see Chapter 1, section 2.3.*), has been shown to inhibit EAE progression while not modulating CNS inflammatory infiltrates and cytokine production[322]. In addition, others have suggested that EAE progression is associated with a marked increase in BVR expression in CNS- infiltrating Mø, astrocytes and neurons[323], suggesting that cells are equipped with the enzymatic machinery necessary to convert BV into BR locally. A possible explanation for

the apparent discrepancy between our results using BV and those obtained with BR is that BR is highly lipophilic and can cross cell membranes while BV is hydrophilic and cannot cross the BBB and thus gain access to the CNS parenchyma. Another possibility is that the protective effect of HO-1 expression within the CNS might also operate via the reduction of cellular labile Fe shown to suppress EAE progression[324], or by limiting its availability to partake in the fenton reaction (*see below*).

The mechanisms underlying the protective effects of HO-1 are associated with both inhibition of T_H and $CD8^+$ T cell accumulation and reactivation within the CNS (*see Chapter 2, section 3.2.*). HO-1 induction by CoPPIX suppressed T_H and CD8+ T cell proliferation as well as IL-2 production by CNS infiltrating T_H cells. In addition, the profile of cytokine expression associated with re-activation of T_H cells within the CNS was also modulated by HO-1 induction in that expression of IFN-γ, but not of IL-10 and TNF-α, was reduced in CNS-infiltrating T_H cells. As such, HO-1 seems to operate by limiting the expansion autoreactive T_H cells as well as by inhibiting the secretion of prototypical T_H1 cytokines, such as IFN-γ, involved in the establishment of demyelination and tissue damage. More recently, however, the relative importance of IFN-γ-secreting T_H1 cells in the pathogenesis of autoimmune neuroinflammation has been questioned as T_H17 cells have been identified as the main pathogenic T cell effector lineage responsible for tissue damage associated with the development of EAE (*see Chapter 1, section 3.1.7.*). Nonetheless, albeit the fact that disease severity is greatly reduced, EAE can still be induced in IL-17-deficient mice[208]. In addition, making use of bone marrow chimeras, it was shown that lack of IL-23 production in microglia, a cytokine crucial for the final differentiation and maintenance of T_H17 cells, did not impact on CNS inflammation, as assessed by the number of CNS-infiltrating cells, but was crucial for the clinical manifestations associated with EAE[224]. Taken together, these observations suggest that although IL-17-producing cells are critical in the effector phase of disease, they might not play a role in disease initiation. On the other hand, T_H1 cells might regulate the initial extravasation of encephalitogenic T_H cell clones into the CNS parenchyma by producing pro-inflammatory cytokines such as IFN-γ, a key molecule in the induction of adhesion molecules associated with EC activation. It is possible, therefore, that by inhibiting the production of IFN-γ by T_H cells, HO-1 might indirectly block the expression of adhesion molecules in ECs of the BBB.

Such an effect might preclude the accumulation of encephalitogenic T_H cells (*see Chapter 1, section 3.1.5.*) and the concomitant local generation of T_H17 cells, responsible for tissue destruction, within the CNS. It is also possible that HO-1 might directly affect the development and/or function of T_H17 cells, a hypothesis that remains to be tested.

Concurrently with the modulation of encephalitogenic T_H cells effector function within the CNS, the protective effect of HO-1 induction could also act via modulation of T_{reg} activity, a cell subset critically involved in the control of autoimmune and inflammatory responses (*see Chapter 1, sections 1.2.1. and 3.1.9.*). However, we found that pharmacological induction of HO-1 expression failed to influence the frequency of $FoxP3^+$ T_{regs} cells within the CNS of mice undergoing EAE suggesting that the protective effect of HO-1 does not act via modulation of T_{regs} function (*see Chapter 2, section 3.3.*). Several reports have suggested that HO-1 expression in T_{regs} is functionally involved in the immune regulatory function of these cells[325, 326]. Based on these reports we analysed the role of endogenous expression of HO-1 in the homeostatic control of T_{reg} maintenance, development and function (*see Chapter 3*). Frequency of T_{regs} in the thymus, spleen and LN was similar in *Hmox-1*$^{+/+}$ and *Hmox-1*$^{-/-}$ mice, indicating that T_{reg} development (thymus) and peripheral maintenance (spleen and LN) are not dependent on HO-1 expression, in either T_{reg} or any other cell type. These findings are in keeping with the observation that HO-1 deficiency is not associated with severe systemic lymphoproliferation, such as it is observed upon null mutation of genes essential for T_{reg} function, namely IL-2, IL-2R and Foxp3[327]. Furthermore, *in vitro* and *in vivo* assays failed to demonstrate any impact of HO-1 expression in T_{reg} activity, suggesting that a functional HO-1 allele in either T_H cells or T_{regs} is not essential for mouse T_{reg} activities. However, the possibility of a functional interplay between HO-1 expression and T_{reg} activity towards immune regulation has not been excluded by these studies. Expression of HO-1 in DCs (*see Chapter 2, section 3.4.*) might affect T_{reg} activity and/or expansion. On the other hand, several observations suggest that T_{regs} can directly inhibit the up-regulation of co-stimulatory molecules or induce the immunomodulatory molecule IDO on DCs suggesting that regulation afforded by these cells is mediated, at least partially, by the modulation of APCs activity[328, 329]. As such, a possible scenario would be that, under inflammatory conditions, T_{regs} could up-regulated the expression of HO-1 in APCs, thus modulating their activity as shown in Chapter 2 and limit the activation of T_H cells. This hypothesis remains to be tested.

The establishment of neuroinflammation during the course of EAE depends critically on the ability of a perivascular-associated DC population to reactivate peripherally primed encephalitogenic T cell clones upon entry into the CNS (*see Chapter 1, section 3.1.6.*). Similarly, during EAE progression naive T_H cells enter the inflamed CNS and are activated by local APCs, possibly DCs, to initiate epitope spreading (*see Chapter 1, section 3.1.6.*). Pharmacological induction of HO-1 expression after EAE onset induced the expression of high levels of HO-1 by CNS-associated DCs. HO-1 would, therefore, modulate the reactivation of encephalitogenic T_H cell activation within the CNS, via modulation of DC activation, and impact on the initial events leading to the establishment of neuroinflammation. Furthermore, by modulating microglia and Mø activation, HO-1 would preclude these cells to sustain inflammation within the CNS and the development of demyelinating lesions (*see Chapter 1, section 3.1.8.1.*). Our data suggests that the impact of HO-1 and/or CO in the downmodulation of encephalitogenic T_H cell activation and effector function is mediated, at least partially, via inhibition of MHC class II expression in APCs, including DCs, microglia and Mø. MHC class II molecules are of central importance in eliciting effector autoimmune T_H cell responses, such as the ones involved in the pathogenesis of EAE. This notion is supported by the observation that interference with MHC class II-mediated antigen presentation to myelin-reactive T_H cells is very efficient in arresting EAE[330, 331].

MHC class II genes are constitutively expressed in APCs, such as DCs and B cells, and can be induced in a plethora of other cell types by several agonists, including IFN-γ. Expression of MHC class II is regulated transcriptionally by the concerted action of several transcriptional factors, including regulatory factor X (RFX), cyclic-AMP-responsive-element-binding protein (CREB) and nuclear transcription factor Y (NFY), that bind consensus DNA sequences in the MHC class II gene promoter. This multiprotein complex, known as MHC class II enhanceosome, constitutes a platform to which class II transactivator (CIITA) is recruited onto the MHC class II promoter. Although not directly bound to DNA, CIITA is thought to promote gene transcription by interacting with general transcription machinery[332] as well as by altering chromatin conformation via histone acetylation or methylation[333, 334]. Contrasting with the ubiquitous synthesis of the MHC class II enhanceosome components, CIITA expression is highly controlled reflecting its role as a master regulator of MHC class II expression. Silencing of CIITA expression is sufficient to abolish MHC class II synthesis in

several cell types[335-337]. Conversely, both constitutive and IFN-γ-induced expression of MHC class II molecules are strictly dependent on CIITA expression[338-340].

Induced expression of MHC class II molecules via IFN-γ involves the activation of a signalling transduction pathway initiated via the IFN-γR and leading to the activation of tyrosine kinases of the Janus kinase (JAK) family that target signal transducer and activator of transcription (STAT)-1. Once activated (phosphorylated), STAT-1 dimerizes and translocates to the nucleus where it binds to the CIITA promoter and activates its transcription. We found that pharmacological induction of HO-1 inhibits STAT-1 activation by IFN-γ with a concomitant reduction in CIITA expression in microglial cells, an effect likely to explain the observed inhibition of MHC class II in these cells (*see Chapter 2, section 3.5.*). The molecular mechanism via which HO-1 inhibits STAT-1 phosphorylation remains to be established. Activation of the JAK/STAT signalling pathway is regulated by the intracellular redox state, i.e. STAT-mediated transcription is sensitive to antioxidants[341-343]. In keeping with this notion NADPH oxidase has been shown to regulate the activation of the STAT signalling pathway[342, 343]. Both HO-1 and CO can act as antioxidants by targeting the expression/activity of NADPH oxidase (*see Chapter 1, sections 2.3. and 2.3.1.*). One likely scenario would be that HO-1/CO targets the heme group of the $gp91^{phox}$ in the NADPH oxidase complex, an effect shown to down modulate the ability of this protein complex to sustain ROS production (*see Chapter 1, section 2.3.1.*). As such, a reduction in STAT-1 activation in APC could be accounted for by a decrease in ROS production due to HO-1 expression and/or activity.

While probably important to the overall protective effect of HO-1, the control of the JAK/STAT-1-dependent expression of MHC class II cannot fully account for the observed inhibition of MHC class II surface expression in DCs. This is suggested by the observation that MHC class II expression associated with DC maturation can occur in a manner that is independent of STAT-1, as demonstrated using STAT-1-deficient mice[344]. Moreover, DCs constitutively express CIITA and consequently MHC class II molecules. Upon DC maturation CIITA is rapidly inactivated at the post-translational level and MHC class II transcription is shut down. During maturation, regulation of MHC class II expression on the cell surface of DCs relies mostly on regulation of protein trafficking to the cell surface and in the controlled rate of MHC/peptide complexes internalisation and

consequent endosomal retention and/or degradation. According to this notion, immature DCs retain MHC class II in the endosomal compartment while activated DCs display most of the MHC class II molecules in the cell surface[345]. Several hypotheses can be put forward to explain the ability of HO-1/CO to control MHC class II surface expression on DCs. HO-1/CO might affect the integration of incoming signals leading to DC maturation, such as the ones provided by TLRs. The engagement of these receptors results in increased antigen loading onto the MHC class II molecules and their redistribution from the endosomal compartment to the cell membrane. Given that HO-1-derived CO limits TLR4-dependent signalling in Mø (*see Chapter 1, section 2.3.1.*), it is reasonable to assume that CO can have similar effects on DCs, therefore influencing MHC class II surface distribution.

There are additional mechanisms via which HO-1 may downmodulate MHC class II surface expression in DC. It is possible that HO-1/CO could impact directly on the mechanisms that regulate membrane dynamics and protein delivery to the plasma membrane. One of such mechanisms is suggested to be inactive in immature DCs due to caspase-mediated proteolitic cleavage of several members of the molecular machinery involved in endosomal transport pathways, namely members of the AP-1 and AP-2 adaptor protein complexes[346]. During DC maturation, inhibition of caspase activity correlates with increased accumulation of functional, full-length protein members of the AP-1 and AP-2 complexes and activation of these transport pathways. Such control of caspase activity during DC maturation is proposed to be iNOS dependent in that NO production is correlated with caspase activity inhibition, suggesting that NOS-derived NO has a role in the control of endosomal trafficking involved in MHC class II molecules redistribution during DC maturation[346]. Like NADPH oxidase, NOS are heme-containing enzymes whose activity can be modulated by HO-1-derived CO (*see Chapter 1, section 2.3.1.*). As such, by decreasing NO production, CO would maintain caspase activity and negatively regulate the endosomal displacement of MHC class II molecules from the endosomes to the cell surface.

The control of MHC class II expression in DCs by HO-1 or CO is of particular relevance. As such, downmodulation of MHC class II expression in DCs that express HO-1 or are exposed to CO might limit T_H clonal expansion and differentiation towards an encephalitogenic phenotype. A corollary of these findings is that HO-1 expression in DCs might regulate the capacity of DCs to elicit T_H cell-mediated responses. If proven correct this effect of HO-1 may be important to explain

its protective effects not only in the context of autoimmunity but also in infectious diseases. In Chapter 4 we address the role of HO-1 and CO in preventing neurological complications resulting from malarial infection, i.e. ECM. Our data demonstrates that expression of HO-1 and its enzymatic activity counters the pathogenesis of ECM, as otherwise resistant mice strains become susceptible to ECM when lacking the *Hmox1* gene (*see Chapter 4, section 3.1.*). We also demonstrate that pharmacological induction of HO-1 expression/function or administration of exogenous CO can be used therapeutically to suppress the pathogenesis of ECM in susceptible mouse strains (*see Chapter 4, section 3.2.*). Furthermore, we have gathered evidence suggesting that HO-1 can limit T cell-mediated immune reactions that contribute in a significant way to the pathogenesis of ECM and associated fatalities. Two observations support this notion. First, *Hmox1*$^{-/-}$ mice in the SCID background, i.e. lacking both mature B and T cells, fail to develop ECM (*data not shown*). Further, depletion of CD8^{+} T cells using an anti-CD8 monoclonal antibody was sufficient *per se* to prevent the onset of ECM in *Hmox1*$^{-/-}$ BALB/c mice (*see Chapter 4, section 3.3.*). Taken together, these observations suggest that HO-1 counters the pathogenic role of T cells, and in particular of CD8^{+} T cells, in triggering ECM. Second, the ability of HO-1 induction or CO exposure to suppress the onset of ECM was associated with decreased number of total CD8^{+} T cells, including activated CD8^{+}CD69^{+} and IFN-γ-secreting CD8^{+} T cells in the CNS (*see Chapter 4, section 3.3.*). HO-1 induction or CO exposure also inhibited the recruitment of innate immune cells, namely monocyte/Mø and polymorphonuclear cells (*data not shown)*. This suggests that, as for EAE, induction of HO-1 expression limits local inflammation and the deleterious immune response associated with the pathogenesis of ECM. Although the mechanism by which such modulation occurs remains to be clearly elucidate, several hypothesis can be put forward.

The role of T_H and CD8+ cells in the pathogenesis of ECM is well established (*see Chapter 1, section 3.2.*). However, the relative contribution of specific APC populations to the initiation of this pathology has remained unclear until recently. The requisite for B cells as APCs can probably be dismissed as B cell-deficient mice exhibit similar incidence of ECM following malarial infection when compared to controls[294]. Making use of mice genetically engineered to enable the selective depletion of conventional CD11c^{+} DCs, this particular cell subset was identified as the sole APC responsible for the activation of T_H cells leading to the onset of ECM[347]. Given the

central role of DCs in promoting deleterious T cell responses in the context of ECM, it is possible that the modulation of DC function afforded by induction of HO-1 expression, identified in EAE, could also contribute to the overall protective effect of HO-1 during the onset of neurological damage associated with malarial infection.

The dependency of T_H cell responses for the establishment of ECM is underscored by the observation that antibody-mediated depletion of $CD4^+$ T_H lymphocytes prevents the onset of disease. These cells are thought to contribute to ECM onset via the production of pro-inflammatory mediators. Moreover, T_H cells can also account for the generation of a primary effector CD8+ T cells, known to be critical for the pathogenesis of ECM (*see Chapter 1, section 3.2.*). This process involves licensing of DCs, where CD40-CD40L signals provided by T_H cells in an antigen-restricted manner allow DCs to became immunogenic, i.e. fully competent to activate naïve CD8+ T cells[348, 349]. As such, it is possible that inhibition of MHC class II expression via expression of HO-1 in DC and concomitant impact on the activation and effector function of T_H cells identified in the context of EAE, can be extended to ECM and explain, at least partially, the observed protection from ECM afforded by HO-1 induction or exposure to CO.

In addition to a T_H cell-dependent activation of $CD8^+$ T cells, HO-1 might impact directly on the generation of effective $CD8^+$ T cell responses. Such could be accomplished via the modulation of antigen cross-presentation, a mechanism by which processed antigens internalised by DCs are presented to CD8+ T cells in the context of MHC class I molecule. Efficient cross-presentation requires the thigh control of antigen processing before loading onto MHC class I molecules, as enhance proteolytic activity would result in the destruction of potential peptides for T cell recognition. Such control is accomplished by regulating pH in endosomes and lysosomes. In mature DCs, NADPH oxidase is recruited to these vesicles sustaining phagosomes alkalinization in a manner that controls the activation of proteases and guarantees the balance between the proper processing of antigens and complete degradation of peptides into aminoacids[350]. In keeping with this notion, $gp91^{phox}$-defective DCs exhibit increased phagosomal acidification and antigen degradation, which relates to a defect in cross-presentation to CD8+ T cells[350]. It is possible, therefore, that when HO-1 is expressed in DCs, the CO generated via heme degradation would target the heme groups in the $gp91^{phox}$ catalytic subunit of the NADPH oxidase blocking its activity. This could impact negatively on antigen cross-presentation and account for the observed reduction

of activated $CD8^+$ cells in the CNS of CoPPIX- and CO-treated animals undergoing malarial infection (*see Chapter 4, section 3.3.*).

Although the reduction of $CD4^+$ or $CD8^+$ T cell accumulation and inflammation in the CNS may be sufficient *per se* to justify the protective effect of HO-1/CO in the development of EAE as well as that of ECM, our data demonstrates the existence of a novel mechanism by which HO-1 controls at least one of this pathologic conditions. As shown for ECM, CO suppresses the generation of free heme and limits BBB disruption (*see Chapter 4, sections 3.4. and 3.5.*). In the course of malarial infections, invasion/replication/egress of RBC by *Plasmodium* leads to extensive hemolysis and concomitant release of Hb into the circulation. Cell-free Hb dimerizes spontaneously and is readily oxidized into MetHb in the presence of ROS. This latter process will lead to the release of heme. Accumulation of free heme in circulation is directly involved in the pathogenesis of ECM as cell-free heme promotes BBB disruption, possibly by catalysing the formation of ROS. This effect, coupled with the presence of activated $CD8^+$ T cells triggers the onset of ECM. When CO binds to cell-free Hb inhibits its oxidation and so the generation of circulating free heme. As such, CO limits BBB leakage and consequent neuronal damage associated with the onset of ECM. While the effects of HO-1/CO in limiting $CD8^+$ T cell accumulation in the CNS and the availability of free heme may act in a concerted way to afford protection against the development of ECM, the latter effect is likely to represent a general mechanism by which HO-1/CO controls the potentially hazardous contribution of free heme, generated under pathophysiology conditions, for the generation of ROS (*see Chapter 1, section 2.2.*). It is conceivable that, similarly to ECM, increased concentrations of free heme or, perhaps more importantly, free heme accumulation within the CNS can account for the intracellular mobilization of redox-active Fe contributing therefore for oxidative damage (*see Chapter 1, section 2.2.*) as it occurs during MS progression (*see Chapter 1, section 3.1.8.1.*). This hypothesis remains however to be tested.

There is ample evidence for a deleterious role of Fe in autoimmune neuroinflammation, which supports a potential pathogenic role of heme in this pathologic condition. Abnormal iron deposits are present in the tissues of EAE-afflicted mice and MS patients[118, 351] and chemical Fe chelation was associated with improved clinical course of disease in experimental systems[352]. While in the context of ECM extensive hemolysis provides a putative source of free heme, that is not the case for EAE and/or MS, as haemorrhage and consequent RBC lysis is a relatively rare

occurrence. Histochemical analysis of cellular Fe distribution within the CNS demonstrates that Fe is localized mainly to the cytoplasm of ODCs where it is likely to be bound to ferritin and transferrin[353, 354]. During immune mediated attack of the CNS the deleterious effects of Fe might therefore be mediated by its release from ferritin such as it should occur upon oligodendrocyte injury. However, it is likely that heme-containing molecules released upon tissue injury provide another potential sources of oxidative damage-promoting Fe. The question then becomes where would free heme be released from as to promote CNS injury in the context of EAE. Similarly to Hb, when exposed to RNS, and possibly to ROS, cytochromes can release their heme prosthetic group[355]. Given the fact that cytochromes are expressed in virtually all cell types and therefore in the CNS as well, they might constitute a putative source of free heme. Another possible source of free heme that could play a deleterious role in the context of EAE as well as MS might be provided by MBP itself, one of the proteins that can elicit self-reactive T_H cell responses leading to the development of autoimmune neuroinflammation. MBP possess a heme-binding domain[356, 357], making it possible that myelin destruction during disease progression could constitute a potential source of free heme. Considering the elevated levels of ROS and RNS in the demyelinating lesion microenvironment due to macrophage and microglia activation (*see Chapter 1, section 3.1.8.1.*), it is possible that heme can be readily oxidised and displaced from heme proteins expressed in the CNS. The observation that free heme can easily assess the intracellular milieu (*see Chapter 1, section 2.2.*), makes it possible that a pathologic feedback loop could be generated where free heme would promote further generation of ROS and concomitant tissue destruction, an effect that we have shown to occur in ODCs (Rafaella Gozellino, IGC, *unpublished observation*). Given that some of the major components involved in the effector response leading to CNS injury, including RNS and ROS can up-regulate the expression of HO-1, by locally producing CO, HO-1 could, as shown for Hb, prevent heme proteins from releasing heme and thus prevent the oxidative stress injury that characterizes the development of neurological lesions associated with the progression of EAE. If proven correct, this could explain the protective effects of HO-1 both in EAE and ECM. According to this notion, the main protective effect of HO-1 would be to counter the deleterious effects of free heme generated during neuroinflammation. To address if this is the case the levels of free heme in the CSF of mice undergoing EAE or those of patients suffering from MS should be evaluated. If elevated levels of free heme are indeed correlated with disease progression, limiting

the potential deleterious role of free heme in promoting target tissue damage should impact on the clinical signs associated with neurological lesion formation. In addition, the control of free heme availability by HO-1/CO could also modulate innate cell activity and production of pro-inflammatory mediators during autoimmune neuroinflammation. This notion is supported by the observation that heme triggers the production of TNF-α by macrophages, an effect dependent on TLR4 activation[358]. In this way, HO-1 activity and/or CO could contribute not only to dampen the inflammatory reaction but also to counter TNF-α-mediated ODC death.

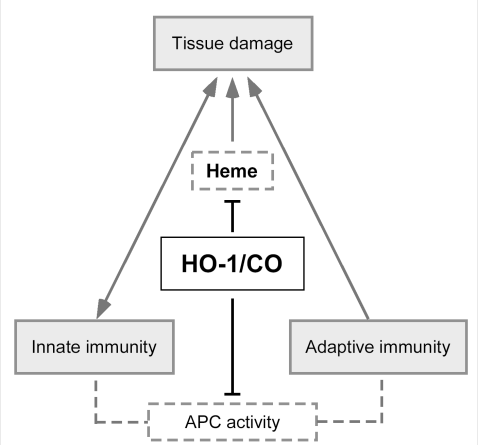

Figure 5.1 – HO-1 and regulation of neuroinflammation. By altering the activation and function of APCs, HO-1 and/or CO modulate deleterious adaptive immune responses involved in the establishment of CNS pathologies, thus limiting the inflammatory response and consequent tissue damage. Moreover, CO generated via heme degradation by HO-1 limits the availability of potentially hazardous free heme, a critical mechanism for the establishment of CNS injury.

In conclusion, we identified in this thesis novel mechanisms by which HO-1 and its products resulting from heme catabolism, more specifically CO, exert potent protective effects in the context of neuroinflammation being it originated from an autoimmune response (MS) or by an infectious agent (CM)(Figure 5.1.). By interfering with these critical mechanisms in the development of inflammatory and immune reaction, we suggest that the protective role of HO-1 and CO identified might be extended to other pathologies and should help designing novel therapies for the treatment of inflammatory disorders.

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Annex 1: Heme Oxygenase-1 modulates the allo-immune response by promoting activation induced cell death of T cells.

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