Neuroprotection by Erythropoietin

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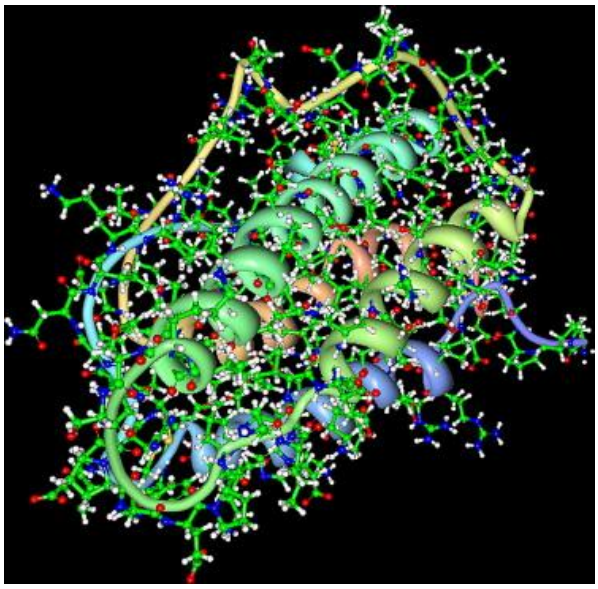

Abstract

Erythropoietin (EPO) is an erythropoietic cytokine that is also neuroprotective *in vitro* and *in vivo*. Neither the mechanism of action of EPO in neuroprotectio, nor the receptor involved is completely known. In fact, EPO regulates erythropoiesis by the homodimeric EPO receptor $(EPOR)_2$. Variants of EPO, not binding $(EPOR)_2$, are still neuroprotective, therefore another receptor may mediate this effect. *In vivo,* EPO is anti-inflammatory in several models of disease but, to date, a direct anti-inflammatory effect *in vitro* has not been clearly found.

The focus of this thesis work was a twofold. Firstly, a direct anti-inflammatory effect of EPO was investigated *in vitro*. It was confirmed that EPO did not have any effect on production of cytokines induced by LPS. In addition, EPO did not reduce cytokines induced by alarmins and other inflammatory stimuli. EPO did not inhibit the pro-inflammatory receptor TREM-1. Finally, EPO did not act as anti-inflammatory by mobilization of endothelial precursor cells *in vivo*.

The second focus of this thesis work was the study of a possible role of EPO on myelination by analysing the induction of myelin genes during differentiation of an oligodendrocyte cell line. EPO upregulated myelin gene expression (MOG and MBP), as studied by qPCR and Western Blot. EPOR was required for the effect of EPO, observed only in cells overexpressing EPOR. EPO induced high levels of the early growth response gene EGR2 that was however not involved in myelin gene induction. Finally, EPO was unable to induce myelin genes in an *in vivo* model of demyelination induced by cuprizone, neither at the peak of demyelination (3 and 5 weeks) nor during the recovery phase.

Greater understanding of effects and mechanisms of action of EPO in the CNS would be useful to find new therapies promoting repair, for instance in diseases like MS in which no drug is available for that purpose.

Candidate's declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to these or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed:

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Contents

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Commonly used abbreviations:

Abbreviations	Definitions
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
CCL2	Chemokine (C-C motif) ligand 2
CEPO	Carbamylated EPO
CG4	Central glia 4
CG4 EGFP	CG4 with empty lentiviral vector
CG4 WT	Central glia 4 wild type cells
CG4EPOR	CG4 overexpressing EPO receptor
CNS	Central nervous system
CTB	Cell titer blue assay
CZ	Cuprizone: bis cyclohexanone oxaldilhydrazone
DAMP	Danger associated molecular patterns
DM	Differentiation medium
DMEM	Dulbecco’s modified Eagle’s medium
EGR2	Early growth response factor 2
EPC	Endothelial progenitor cells
EPO	Erythropoietin
EPOR	Erythropoietin receptor

Abbreviations	Definitions
FBS	Fetal bovine serum
FCS	Fetal calf serum
GM	Growth medium
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank’s balanced salt solution
HIF	Hypoxia inducible factor
HMGB1	High mobility group box protein 1
HuREPO	Human recombinant EPO
IP	Ischemic preconditioning
LIF	Leukemia inhibitory factor
LPS	lipopolysaccharide
MBP	Myelin basic protein
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MM6	MonoMac6 cells
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NAA	Non-essential amino acid
NCL	Necrotic cell lysate
NPC	Neural precursor cells
OPC	Oligodendrocyte precursor cell

Abbreviations	Definitions
P/S	Penicillin/streptomycin
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGFRα	Platelet-derived growth factor receptor α
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium dodecyl sulphate polyacrilamide gel electrophoresis
TLR	Toll like receptor
TREM-1	Triggering receptor expressed on myeloid cells
βcR	Common β receptor

Chapter 1: Introduction

1.1 Erythropoietin and erythropoiesis

Erythropoietin (EPO) is a 34 kDa glycoprotein consisting of 165 amino acids folded in 4 α-helices with 2 bisulphide bridges. It was originally purified in small amount from urines of aplastic-anemic patients (Miyake et al., 1977). Later in the mid '80s, human recombinant EPO (rHuEPO), with the same amino acid sequence of endogenous EPO, was synthesized and commercialised for anemic patients.

When discovered, EPO was only considered a hematopoietic growth factor required for erythrocytes differentiation. It is produced by fetal liver and adult kidney and induced by hypoxia through the hypoxia-inducible factor (HIF), and therefore improves tissue-oxigenation, when the tissue oxygen concentration is low. EPO specifically increases the number of circulating erythrocytes by promoting the survival of erythroid progenitor cells with an anti-apoptotic mechanism.

EPO effect on erythropoiesis is mediated by binding to the specific homodimeric erythropoietin receptor $(EPOR)_2$. EPOR is a transmembrane receptor that belongs to the single chain cytokine type 1 receptor family that recognizes cytokines with 4 α-helices. These receptors are characterised by an extracellular N-terminal domain with conserved cysteins, a single hydrophobic transmembrane segment and a cytosolic domain with no intrinsic kinase activity. When EPO binds $(EPOR)_2$, there is a conformational change which activates two Janus family tyrosine kinase 2 (JAK2) molecules that associate with a cytoplasmic domain of EPOR. Once JAK2 is phosphorylated, there is subsequent activation of signalling molecules that activate multiple transduction pathways (e.g., STAT, PI3K, NFKB and calcium) (Youssoufian et al., 1993). STAT5, PI3K/Akt and Ras/MAPK pathways appear to mediate the anti-apoptotic action of EPO on erythroid precursor cells. EPO signalling is terminated by

the activation of phosphatases which dephosforylate JAK2. The ligand-receptor complex is then internalised and degraded by the proteasome.

1.2 Erythropoietin and neuroprotection

At first, endogenous EPO was thought to be produced only by fetal liver and adult kidney, but its local production by the central nervous system (CNS) is now well documented.

EPO is expressed in the brain mainly in astrocytes (Masuda et al., 1994; Marti et al., 1996) and oligodendrocytes (Sugawa et al., 2002). EPO receptor (EPOR) was detected in the brain *in vivo* (e.g. hippocampus, cortex, midbrain; Digicaylioglu et al., 1995) and *in vitro* (e.g. neurons, astrocytes, oligodendrocytes, microglia, endothelial cells; Nagai et al., 2001; Noguchi et al., 2007; Sugawa et al., 2002). The expression of EPO and EPOR in the CNS led to the hypothesis of an effect of EPO on neuroprotection that started to be investigated. However, exogenous EPO was not considered able to pass the blood brain barrier (BBB) since it is a high molecular weight cytokine. The demonstration that systemically-administered rHuEPO crossed the BBB stimulated a growing interest in the neuroprotective action of EPO (Brines et al., 2000). The amount of rHuEPO that passed through the BBB was less than 0.1 % in 3 hours following intravenous injection. However, when high doses were administered, EPO crossed the BBB in therapeutic effective concentrations. In fact, it was found in similar amount of other cytokines known to exert effects on the brain by crossing the BBB (Banks et al., 2004). Moreover, the BBB can be damaged in pathological conditions, like in multiple sclerosis (MS), increasing the amount of EPO that crosses the BBB and is available in the brain.

To date, EPO has been shown to have protective actions in various *in vivo* models of injuries and diseases associated with neuronal death, neurodegeneration or neuroinflammation, including stroke (Brines et al., 2000; Brines et al., 2000; Siren et al., 2001a; Villa et al., 2007), brain trauma (Brines et al., 2000), diabetic neuropathy (Bianchi et al., 2004), cisplatin neuropathy (Bianchi et al., 2006), motoneuronal degeneration in wobbler mice (Mennini et al., 2006) and experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Agnello et al., 2002a) (Savino et al., 2006). The clinical relevance of these studies has been

provided by pilot clinical trials showing that EPO administration may be an effective neuroprotective approach in patients with stroke (Ehrenreich et al., 2009), schizophrenia (Ehrenreich et al., 2007b), MS (Ehrenreich et al., 2007a), amyotrophic lateral sclerosis (Lauria et al., 2009) and subarachnoid hemorrhage (Tseng et al., 2009).

Nevertheless the erythropoietic action of EPO may be undesirable in non-anemic patients, where an increase in the hematocrit above normal values increases blood viscosity, blood pressure and the risk of thrombosis. For instance, in the last clinical trial in stroke patients an increase in the mortality rate in the EPO treated group was reported (Ehrenreich et al., 2009).

Finally, some evidence in the last years highlights a role for endogenous EPO as part of the network of cytokines involved in neurological diseases. The induction of endogenous EPO in the CNS was mainly reported for hypoxic/ischemic pathologies. It was shown that endogenous EPO is induced in neurons and astrocytes by hypoxia (Bernaudin et al., 2000) and it is produced following cerebral ischemia (Bernaudin et al., 1999) (Siren et al., 2001b). Endogenous EPO was demonstrated to mediate the phenomenon of the ischemic preconditioning (IP) *in vitro*, when released by astrocytes (Ruscher et al., 2002), and *in vivo* (Prass et al., 2003). The IP is a biphasic phenomenon with a first short phase of protection, developed within minutes from the initial ischemic insult and lasting for less than 2 hours, and a second one 12-72 hours after the preconditioning event and lasting for at least 3 days. In addition, Mengozzi et al. showed that endogenous EPO is also induced in the spinal cord of mice with EAE, the animal model of multiple sclerosis (Mengozzi et al., 2008), and might be part of a protective response. Recently, Kato et al. demonstrated that endogenous EPO released from astrocytes under hypoxia protects oligodendrocyte precursor cells (OPCs) from damage under hypoxic/reoxigenation conditions (Kato et al., 2011).

Therefore, endogenous EPO could have some interesting effects in neuroprotection that need to be further investigated.

1.3 The unknown receptor mediating neuroprotection

An understanding of which receptor mediates the neuroprotective effects of EPO may lead to a new treatment approach using a derivative of EPO that does not possess the unwanted side-effects associated with the erythropoietic action. Thanks to many studies about structure-activity relationship, the regions of EPO interacting with $(EPOR)_2$ were identified: the portions of helices A and C, helix D and the loop connecting helices A and B (see Fig. 1.1).

The literature about the receptor of EPO mediating neuroprotection is controversial. Recently, the functional role of EPOR in non hematopoietic cells was also questioned, although only in one paper (Sinclair et al., 2010), opening a debate on whether EPO has any effect outside the erythropoietic system (Ghezzi et al., 2010). In this regard, Xiong et al. found that EPO reduces cortical tissue damage and hyppocampal cell loss after traumatic brain injury in mice in absence of neural EPOR (Xiong et al., 2010).

However, EPOR is required to mediate EPO effect in neuroprotection (Yu et al., 2002) (Um et al., 2007), EPOR expression is increased during pathological conditions in humans, like in ischemic infarcts and hypoxic brain damage (Siren et al., 2001b) or ex vivo in mice after demyelination induced by lysolecithin (Cho et al., 2012), and its induction also mediates the effect of hypoxia preconditioning (Chen et al., 2010).

Brines et al. demonstrated that derivatives of EPO that do not bind $(EPOR)_2$ are not erythropoietic but still retain tissue-protective activities (Brines et al., 2008), so another receptor is suspected to be involved in neuroprotection. Carbamylated EPO (CEPO) is a derivative of EPO that fails to bind $(EPOR)_2$ and is obtained by carbamylation of all lysines to form homocitrulline. CEPO does not increase the hematocrit in mice but it is protective in animal models of cerebral infarct, spinal cord injury, diabetic peripheral neuropathy and in the mouse model of MS (Leist et al., 2004).

Figure 1.1: Scheme of the EPO/(EPOR)2 interaction (Brines et al., 2008). The regions of EPO molecule binding the dimeric EPO receptor are: helices A and C, helix D and the loop between helices A and B. The aqueous face of helix B faces away from the interior of the receptor.

Another derivative of EPO without hematopoietic effects is the helix B peptide (HBP), synthesized as linear sequence of helix B, that does not bind $(EPOR)_2$ (Brines et al., 2008). HBP is still neuroprotective *in vitro* (kainic acid- induced motoneuron excitotoxic death) and *in vivo* (protection in a stroke model and prevention of retinal edema development in a rat model of diabetes) (Brines et al., 2008). Several other derivatives were synthesized and under investigation for their neuroprotective activities.
The main hypothesis about the receptor mediating the tissue protective effect of EPO is the heterodimer between EPOR and the common β receptor (βcR), also named CD131 (Brines et al., 2004). βcR contains a signal-transducing subunit shared by the granulocyte-macrophage colony stimulating factor (GM-CSF), IL-3 and IL-5 receptors. The βcR is only a subunit of the complete heterodimeric receptor for these cytokines and alone it is not able to bind any of them. Previous studies showed a functional interaction between EPOR and the βcR that is a type 1 cytokine receptor. IL-3, IL-5 and GM-CSF are involved in differentiation and function of myeloid cells but they also have an important role in modulating Th1 and Th2 immune response and in the pathophysiology of some neurodegenerative disorders (Martinez-Moczygemba and Huston, 2003). Therefore, a common mechanism of action between these cytokines and EPO could be supposed. Since βcR knockout mice have a normal erythropoiesis (Scott et al., 2000), βcR is not involved in this function. In addition, the βcR is expressed in neuronal cells; therefore the main hypothesis is that the EPOR monomer interacts with the βcR subunit to mediate neuroprotection. In support of that, CEPO bound this heterodimeric receptor (Brines et al., 2004).
According to Brines, it would be better to speak of a tissue-protective receptor (TPR) that it is not normally expressed by tissues but that is activated during diseases and injuries (Brines and Cerami, 2012) (Fig. 1.2). It should be preferably formed by βcR-EPOR, but whenever EPOR is not co-localised with βcR, it will self associate and form $(EPOR)_2$, which can also support neuroprotection (Brines and Cerami, 2012).

The affinity of EPO for the EPOR/βcR receptor is low; therefore high doses would be necessary to bind this receptor.

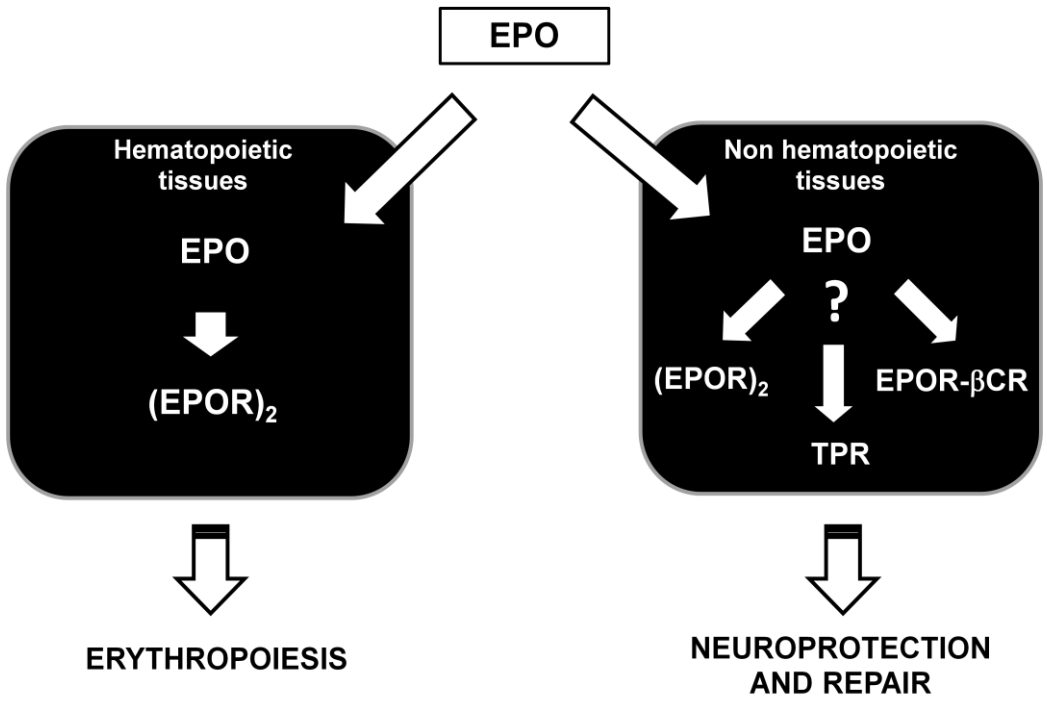


Figure 1.2: Scheme of the different EPO mechanism in erythropoiesis and neuroprotection.

1.4 EPO mechanism in neuroprotection

The mechanisms by which EPO acts in neuroprotection have been investigated *in vitro* and *in vivo*. EPO and EPOR are expressed at basal levels in the brain in normal conditions, and both proteins are greatly induced following ischemic stressors or in pathological conditions, as reported in preclinical (Bernaudin et al., 1999) and clinical studies (Eid et al., 2004). This up-regulation may also be induced by inflammatory cytokines like TNFα, IL1 and IL6 (Nagai et al., 2001; Taoufik et al., 2008).
The action of EPO as a neuroprotective agent may involve several mechanisms *in vivo* such as inhibition of neuronal (Siren et al., 2001a) and oligodendrocyte death (Genc et al., 2006), inhibition of CNS inflammation and associated cytokine production (Villa et al., 2003), induction of other neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Viviani et al., 2005), mobilization of stem cells (Shingo et al., 2001) and augmentation of neurogenesis (Wang et al., 2004). Interestingly, EPO is also able to mobilize endothelial progenitor cells (EPCs) from the bone marrow and affect their proliferation and differentiation. EPCs cells are important in promotion of vascular reparative processes and their stimulation in number and function is related to an improvement in cardiac functions. EPO was able to significantly regulate the number of functionally active EPCs in humans and mice (Bahlmann et al., 2004; Heeschen et al., 2003). Darbepoetin, an EPO analogue, was also able to regulate EPCs proliferation and differentiation with a standard therapeutic dose (Bahlmann et al., 2004).
Summarising, EPO seems to have three main effects: protection from neuronal death, inhibition of inflammation and stimulation of neurorepair. The role of EPO in neuroprotection has been studied also *in vitro* to understand the mechanisms activated by EPO to mediate each effect.

1.4.1 Neuronal protection from death

EPOR is expressed in the brain in neural progenitor cells (NPC), neurons, astrocytes, microglial cells and oligodendrocytes. Several studies reported that EPO has a protective effect against cell death in various hypoxic, hypoglycaemic and excitotoxic *in vitro* models (Table 1.1).
EPO was reported to induce several different protective responses *in vitro*, like protecting cultured neurons against glutamate toxicity that is considered one of the most important mechanisms known to trigger cell death in CNS disorders (Morishita et al., 1997). The main mechanism by which EPO protects neurons is its ability to inhibit apoptosis reducing DNA damage and cell membrane asymmetry. EPO protects from neuronal apoptosis through activation of JAK2/STAT5. By activation of Bcl-XL and Bcl-2 expression, two pro-survival proteins, EPO regulates the balance between pro and anti-apoptotic pathways. EPO also inhibits caspase 3 and caspase 9, involved in mediation of apoptosis. In addition, EPO had also some indirect effect like the increased astrocyte production of glutathione peroxidase that ameliorates neuronal damage caused by excitotoxins (Genc et al., 2002).

Type of neurons	Type of toxicity	Reference
Cortical/hippocampal neurons	Glutamate	(Morishita et al., 1997)
Hippocampal neurons	NO	(Sakanaka et al., 1998)
PC12 cell line	Serum deprivation	(Koshimura et al., 1999)
Hippocampal neurons	Hypoxia	(Lewczuk et al., 2000)
Cortical neurons	NMDA	(Digicaylioglu and Lipton, 2001)
Motor neurons	Kainate	(Siren et al., 2001a)
Hippocampal neurons	Hypoxia	(Siren et al., 2001a)
P19 cell line	Serum deprivation	(Siren et al., 2001a)
Cortical neurons	Oxygen/glucose deprivation	(Ruscher et al., 2002)
Hippocampal neurons	Chemical hypoxia	(Wen et al., 2002)
Hippocampal neurons/endothelial cells	Anoxia	(Chong et al., 2003)
Cortical neurons	Oxygen/glucose deprivation, AMPA	(Sinor and Greenberg, 2000)
PC12 cells	1-Methyl-4-phenylpyridinium	(Wu et al., 2007)
SH-SY5Y	Staurosporine	(Um et al., 2007)

Table 1.1: *In vitro* neuroprotection by erythropoietin.

1.4.2 Inhibition of inflammation

The effect of EPO on inflammation is controversial. In fact, the anti-inflammatory role of EPO has been demonstrated *in vivo* in several models of disease. As mentioned before, in a stroke model obtained by middle cerebral artery occlusion (MCAO), EPO attenuated inflammation by reducing microglia activation (Wang et al., 2007), by inhibiting immune cell recruitment in the injured area and decreasing inflammatory cytokines (Villa et al., 2003). In the MS animal model, the experimental autoimmune encephalomyelitis (EAE), EPO delayed the increase of TNFα and decreased the expression of inflammatory cytokines like IL6, IL1β and IL1α (Agnello et al., 2002a) (Savino et al., 2006); EPO also decreased the up-regulation of the major histocompatibily complex (MHC) in the spinal cord (Li et al., 2004). In addition, EPO decreased the expression of inflammatory cytokines in the neonatal hypoxia/ischemia model (Sun et al., 2005) and in the cerebral malaria model (Kaiser et al., 2006) (Wiese et al., 2008). However, to date, a direct anti-inflammatory effect of EPO was found just in few cases in *in vitro* studies. In fact, the anti-inflammatory effect of EPO *in vitro* seems to be related to an anti-apoptotic effect on neurons more than arising from a direct reduction of pro-inflammatory cytokines. In this regard, Villa et al. found that EPO did not inhibit lipopolysaccharide (LPS)-induced TNFα neither in glial cells nor in peripheral blood mononuclear cells (PBMCs) (Villa et al., 2003). However, in neuron-glial co-cultures exposed to trimethyltin (TMT), a neurotoxin increasing glial TNF production as a consequence of neuronal death from apoptosis, EPO reduced the production of TNF by decreasing apoptosis (Villa et al., 2003). An effect of inhibition of LPS-induced TNF was not even found in murine microglia (Wilms et al., 2009). On the contrary, it was reported that EPO inhibits LPS-induced TNF in U937 (Yazihan et al., 2008) and in peritoneal macrophages or RAW 264.7 cells (Nairz et al., 2011). In conclusion, the mechanism involved in mediation of EPO anti-inflammatory activity is not completely understood.

So far, the anti-inflammatory effect of EPO has been mainly studied *in vitro* on LPS-induced cytokines. Since inflammation can be mediated by several

other factors rather than just LPS, the anti-inflammatory effect of EPO can be studied on other models of inflammation.

All the factors responsible for inducing an inflammatory response are now classified in two different groups of danger signals, known as danger-associated molecular patterns (DAMPs): pathogen-associated molecular patterns (PAMPs), of exogenous origin, and alarmins, that are endogenous molecules (Bianchi, 2007). PAMPs and alarmins evoke similar responses, by activation of pattern recognition receptors among which toll like receptors (TLRs) are the most well known (Akira, 2006). TLR are type I membrane proteins that are key players of the innate immunity and involved in recognition of molecular components of invading bacteria, viruses and parasites (Fig. 1.3).

The prototypical PAMP is LPS, found in the outer membrane of various Gram-negative bacteria, released in septic shock and acting by activation of TLR4. Moreover, in this group there are also bacterial flagellin, acting by TLR5; lipoteichoic acid, found in the cell wall of Gram-positive bacteria, acting by TLR2 and double stranded RNA from viruses acting by TLR3. Among the endogenous mediators, the best known is the high mobility group protein-1 (HMGB1), late cytokine mediator of lethal endotoxemia and sepsis (Yu et al., 2006). HMGB1 extranuclear expression is involved in a number of pathogenic conditions: sepsis (Yang et al., 2004), arthritis (Taniguchi et al., 2003), atherosclerosis (Porto et al., 2006), cancer (Taguchi et al., 2000), stroke (Qiu et al., 2008) . HMGB1 is a nuclear protein, it is released when cells die in a traumatic way (Bianchi and Manfredi, 2007), and can act by TLR2, TLR4 and the receptor for advanced glycation end products (RAGE) (Kokkola et al., 2005). In contrast, apoptotic cells modify their chromatin so that HMGB1 is not released anymore.

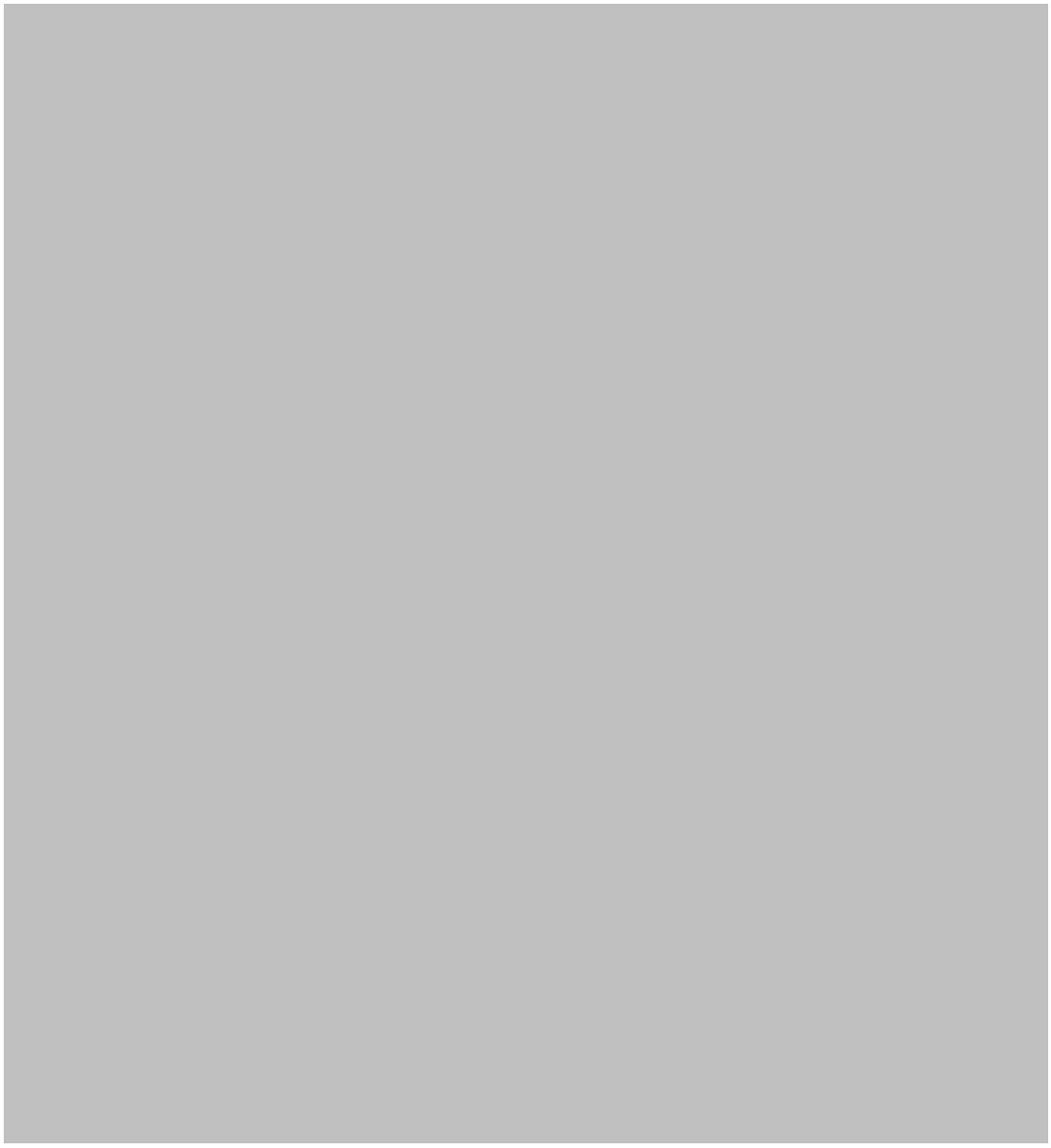


Figure 1.3: DAMPs (PAMPs or alarmins) activate inflammation. The secretion of inflammatory cytokines can be induced by binding to different receptors. RAGE is a multiligand receptor of the immunoglobulin family expressed on macrophages, neurons and endothelial cells. Some DAMPs can engage TLRs to induce and amplify the inflammatory response. TREM-1 is member of immunoglobuline family as well and is expressed in neutrophils and monocytes (www.invivogen.com).

HMGB1 can also be actively secreted by macrophages, monocytes and DCs following different stimuli and can act through different pathways (Klune et al., 2008).

In preclinical studies, HMGB1 alone stimulated an inflammatory response in animals by induction of TNF and IL6 and mediating anorexia and taste aversion (Agnello et al., 2002b). Furthermore, it has been shown that also *in vitro* HMGB1 stimulates pro-inflammatory cytokines in human monocytes (Andersson et al., 2000) and macrophages (Yang et al., 2010).

Other alarmins are: hepatoma-derived growth factor that can be passively released by necrotic cells via a non classical pathway but it is retained by apoptotic cells; heat shock proteins, released by necrotic cells; uric acid released by injured cells; IL1α and several others.

El Mezayen et al. showed that a necrotic cell lysate (NCL) from a human monocytic cell line (THP-1), likely containing a mixture of alarmins, is able to induce TNF-α, IL6 and IL-8 when compared with control medium (El Mezayen et al., 2007). Therefore, another way to induce cytokines *in vitro* by a mechanism mediated by several danger signals might be to treat cells with NCL.

A complex involved in mediation of inflammation is the inflammasome. It is a component of the innate immune system expressed in myeloid cells that can be activated by several inductors. One of these stimuli is the adenosine triphosphate (ATP) that is also considered an endogenous danger signal. In fact, extracellular ATP is a potent stimulus for the inflammasome via the purinergic receptor P2X (P2X7R) that is a cell surface receptor for ATP in macrophages and other immune cells. The inflammasome controls the activation of the enzyme caspase-1 by activating the pro-caspase-1, and subsequently regulates the maturation of IL-1β from the pro-IL1β, induced by some priming stimuli like LPS or TNFα (Franchi et al., 2009).

As reported by Mengozzi et al., in order to investigate the mechanism by which EPO inhibits inflammation, a microarray study was performed in a rat model of cerebral ischemia (Mengozzi et al., 2012). In this model of

brain damage, EPO had clearly protective effects and therefore it was a good system to investigate the mechanism by which EPO acts, studying genes modulation by EPO. In this study, EPO did not have any effect on inflammatory genes, with the exception of the triggering receptor expressed on myeloid cells (TREM-1), that was inhibited by EPO (Mengozzi et al., 2012). TREM-1 is a pro-inflammatory receptor, expressed on neutrophils and $CD14^{high}$ monocytes, involved in the amplification of inflammation in ischemic and autoimmune diseases. It is up-regulated in infections *in vivo* and following TLR engagement *in vitro* (Bouchon et al., 2000).

1.4.3 Induction of neurorepair

EPO modulates neurorepair through different mechanisms like neurogenesis, angiogenesis and oligodendrogenesis.
Neurogenesis is the process by which new neurons are generated from stem/progenitor cells. EPOR is expressed in neurons and EPO has a neurotrophic activity in the cerebral system during development. EPO stimulates the differentiation of pluripotent stem cells to neuronal precursor cells (NPC) and the neural differentiation of NPC.
Angiogenesis is the process of formation of new capillary vessels from the pre-existing vessels, to optimize the oxygen delivery to tissues. EPOR is expressed in endothelial cells in the blood vessels and these cells proliferate in response to the EPO treatment.
Oligodendrogenesis is the production of new oligodendrocyte cells. These cells are neuroglial cells responsible to produce myelin, the fatty protective coating surrounding axons in the CNS, by the myelination process. In the adult brain, oligodendrocytes are produced from OPCs resident in the brain parenchyma or in the subventricular zone. Oligodendrocytes undergo rapid differentiation into mature myelinating oligodendrocytes. In some pathologies, like MS, an acute demyelination occurs during the disease and the remyelination process fails for several, and as yet unknown, reasons (Fig.1.4). Understanding the mechanisms regulating this process of demyelination-remyelination could be useful to find therapies to promote remyelination, since nothing is currently available for that purpose.
A correlation between EPO and oligodendrocytes has been established. First of all, the expression of EPO has also been found in the brain in oligodendrocyte cells, where the level of EPO mRNA is even higher than in neurons and astrocytes (Sugawa et al., 2002). EPOR was detected in oligodendrocytes at the same level as in neural cells (Sugawa et al., 2002). Oligodendrocytes are responsive to EPO, as it was demonstrated that EPO inhibits the expression of iNOS and nitrite production resulting from pro-inflammatory stimulation by IFNγ and LPS in primary rat oligodendrocytes (Genc et al., 2006).

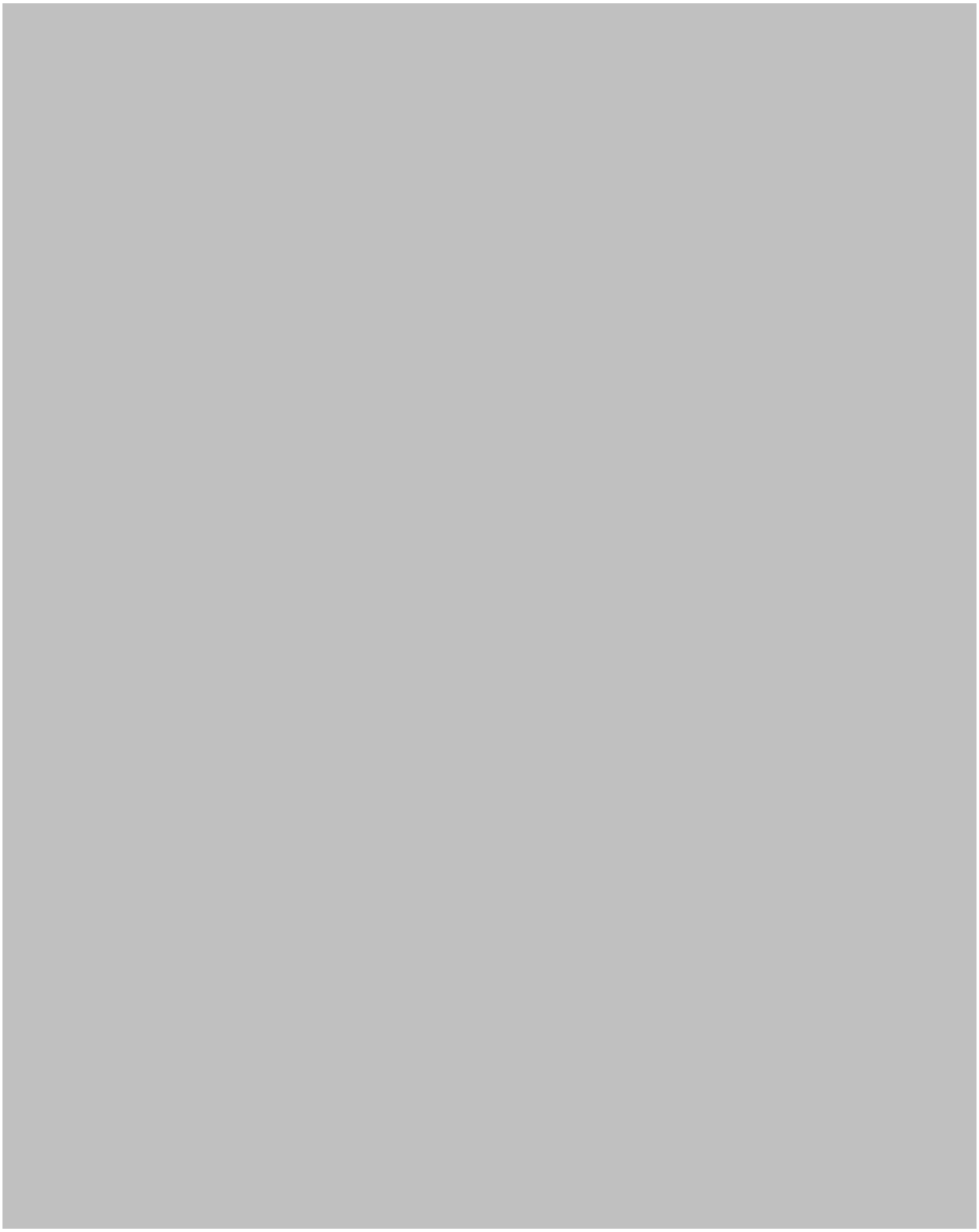


Figure 1.4: Scheme of de-remyelination process (Bruce et al., 2010).

A. CNS axons are myelinated by oligodendrocytes. A single oligodendrocyte can make multiple myelin sheaths (also called internodes).

B. In some diseases, such as MS, the oligodendrocyte and its myelin sheath are the target of pathological process. The death of oligodendrocytes and the myelin sheath it maintains is called demyelination. Note that the axon remains intact.

C. The default response to demyelination is the generation of new oligodendrocytes from a widespread population of stem/ precursor cells called OPCs. These new oligodendrocytes put new myelin sheaths around the demyelinated axons in the regenerative process of remyelination. Note that the myelin sheaths of remyelination are thinner and shorter than the original myelin sheath made during development.

D. In some situations, notably the later stages of MS, remyelination fails, leaving the axons chronically demyelinated. In this state they are very vulnerable to atrophy, a degenerative response from which there is little spontaneous recovery.

In addition, the hypoxia-induced cell damage of OPCs is suppressed by endogenous EPO produced by astrocytes (Kato et al., 2011).
In vivo EPO clearly showed a protective effect in experimental autoimmune encephalomyelitis (EAE), the MS animal model, by decreasing disease severity (Savino et al., 2006).
In this model EPO had an anti-inflammatory role by diminishing the inflammatory cell infiltration in the spinal cord of EAE rats (Agnello et al., 2002a) (Zhang et al., 2010) and the expression of inflammatory cytokines (Savino et al., 2006).
Recently, a role of EPO in repair was also hypothesized since few studies correlate EPO with oligodendrogenesis. EPO attenuated white matter injury in neonatal rats following hypoxia/ischemia (Iwai et al., 2010); increased OPCs proliferation (Zhang et al., 2005) and increased the number of myelin basic protein (MBP)-positive cells in oligodendrocyte cultures (Sugawa et al., 2002). MBP is a major component of myelin as well as myelin oligodendrocyte glycoprotein (MOG) that is also mainly expressed after the onset of the myelination process (Solly et al., 1996). These two genes can be considered markers of oligodendrocytes differentiation, since they are produced only by mature oligodendrocyte cells.
Finally, Cho et al. recently demonstrated an effect of EPO in an *in vivo* model of demyelination induced by lysolecithin. EPO induced myelin repair, as examined by Luxol Fast blue histochemistry and immunohistochemistry for MBP (Cho et al., 2012). One interesting result obtained in that study was that EPOR was found up-regulated after lysolecithin-induced injury and that an increase in the number of OPCs with up-regulated-EPOR expression was demonstrated. Increased expression of EPOR could be important for a higher EPO-mediated oligodendrogenesis in OPCs (Cho et al., 2012).
An *in vitro* model that has been used several times and that is considered a good tool to study myelination is the Central Glia-4 cells (CG4). These cells are a rat line of oligodendrocyte precursors originally obtained from primary cultures of bipotential oligodendrocyte-type-2-astrocyte (O-2A) progenitor cells. CG4 cells, as the same of O-2A cells, are able to

differentiate into oligodendrocytes or type-2-astrocytes depending on culture conditions. By withdrawal of B104 mitogens and also of bFGF and PDGF, cells differentiate into oligodendrocytes. Mitogens replacement with fetal calf serum (FCS 20%) induces differentiation to type-2-astrocytes. CG4 cell differentiation is characterized by acquisition of branched membrane processes (Louis et al., 1992). At the later stage of differentiation CG4 cells express myelin genes like MOG and MBP. Morphologically undifferentiated cells look bipolar and they acquire a >90% of multipolar morphology after 2 days of differentiation.

The main model to study remyelination *in vivo* is the cuprizone-induced demyelination, a model with minor inflammation localised to the brain. Cuprizone, bis-cyclohexanone oxaldihydrazone (CZ), induced microscopic lesions in the brain accompanied by edema, hydrocephalus, demyelination and astrogliosis. CZ is a copper chelator that induces demyelination specifically in the brain and predominately in the white matter (corpus callosum and cerebellar peduncles). CZ acts mainly on oligodendrocytes causing their damage and death (Matsushima and Morell, 2001), probably by microglia activation. However, the cuprizone mechanism is not fully understood yet. Between 3 and 5 weeks of CZ diet feeding, levels of myelin gene expression are dramatically decreased (Morell et al., 1998) and the corpus callosum is almost completely demyelinated. In this model, acute demyelination is followed by spontaneous remyelination during subsequent weeks when mice are fed with normal chow (Morell et al., 1998).

1.5 Aim of the study

The aim of this study was to understand more about the mechanisms that mediate EPO effectiveness in animal models of MS, in particular in EAE, in which the disease pathogenesis is mediated by neuroinflammation and demyelination.
The main specific hypotheses were:

1. EPO could inhibit the production of inflammatory cytokines *in vitro*.
2. EPO could increase the expression of myelin genes.

To test these hypotheses, the study was divided in two sections:

1. **Investigate whether EPO inhibits the production of inflammatory cytokines *in vitro***. Since EPO inhibited inflammatory cytokines *in vivo* in EAE but controversial results had been obtained *in vitro* on LPS-induced cytokines, we hypothesized that EPO might inhibit inflammatory cytokines induced by different pathways rather than LPS, in particular by endogenous danger signals like HMGB1. For these experiments, we used a line of human monocytic cells and also primary human PBMCs and macrophages.

2. **Investigate whether EPO increases myelin gene expression.** For these experiments, we used rat CG4 cells, a line of oligodendrocyte precursors widely used as a model of myelination *in vitro*. The availability of the same cell line transduced to overexpress EPO receptor allowed us to specifically study the involvement of the classical EPOR in this EPO effect. We also investigated whether EPO increases myelin gene expression in an *in vivo* model of chemically-induced demyelination, the cuprizone model.

Chapter 2: Materials and methods

2.1 Materials

Product name	Supplier
Absolute Blue QPCR SYBR Green Mix	Thermo Scientific
Apo-transferrin	Sigma-Aldrich
b FGF	Invitrogen
Biotin	Sigma-Aldrich
Brilliant II QPCR-Master Mix	Agilent technologies
Chanazine 2%	Chanelle Animal Health
Clorophorm	Sigma-Aldrich
CTB cell viability assay	Promega
Cuprizone	Sigma-Aldrich
DMEM	PAA Laboratories
DMEM-F12	Sigma
DMSO	Fisher-Scientific
DNA primers and probes for Taqman PCR	Applied Biosystem
DNA primers for SYBR Green PCR	Invitrogen
dNTP	Techne
DTT	Invitrogen
Dulbecco PBS without Ca & Mg	PAA Laboratories
ELISA Duoset kits	R&D Systems
Fetal bovine serum (FBS)	Invitrogen

Product name	Supplier
Fetal calf serum (FCS)	Invitrogen
Glucose	Sigma-Aldrich
Glutamine	Invitrogen
HBSS	GE Healthcare
hPDGF-AA	Invitrogen
Hu recombinant EPO	Dragon Pharmaceuticals
Hu recombinant HMGB1	Provided by Kevin Tracey
HyPure molecular biology grade water	Thermo-Scientific
IL-1α	Peprotech
IL-1β	Peprotech
Insulin	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Ketaset 100	Fort Dodge Animal Health
KU 0063794	Tocris
Lipofectamine RNAiMAX	Invitrogen
LPS	Sigma-Aldrich
Lympholyte-H	Cederlane
M-CSF	eBioscience
M-MLV-RT	Invitrogen
N1 supplement	Sigma-Aldrich
NaCl	Aquafarm
NEAA	GIBCO
OPI - Supplement	Sigma-Aldrich

Product name	Supplier
PAM3cys	Alexis
PBS/Citrate	Fisher
Pen/strep	GIBCO
Percoll™ - PLUS	GE Healthcare
Plates and flasks for CG4 cells	Falcon
Poli-L-ornithine	Sigma-Aldrich
Progesterone	Sigma-Aldrich
Putrescine	Sigma-Aldrich
R848	Invitrogen
Random Primers	Promega
RNAse OUT	Invitrogen
RPMI-1640	PAA Laboratories
RT buffer	Invitrogen
Silencer Select Pre designed siRNA	Ambion
Sodium selenite	Sigma-Aldrich
Soy bean trypsin inhibitor	Sigma-Aldrich
Staphylococcus	Provided by Charles Dinarello
Thyroxin	Sigma-Aldrich
TRIzol reagent	Invitrogen

2.2 *In vitro* models

2.2.1 MonoMac6 cells (MM6)

MonoMac6 (MM6) cells are a human monocytic cell line.
These cells were maintained in RPMI-1640 medium supplemented with 0.2% penicillin/streptomycin (P/S), 1% glutamine, 1% OPI-supplement (oxaloacetate, pyruvate and insulin), 1% non-essential amino acids (NEAA) and 10% fetal bovine serum (FBS). For the experiments, MM6 cells were plated at a concentration of 4 X 10^5 cells/well in 24 well plates and treated with different concentrations of HMGB1 or EPO. Cell-free supernatants were collected, by centrifugation in microcentrifuge at 10,000 rpm for 3 minutes, for ELISA assays. For RNA extraction cells were harvested with TRIzol reagent.

2.2.2 Peripheral blood mononuclear cells (PBMCs)

Peripheral blood was purchased from the National Health Service Blood and Transplant (NHSBT). All donors gave written informed consent. The study was approved by the local ethics committee (R&D Ref. no: 09/168/SAC).
Blood was diluted with Hank's balanced salt solution (HBSS) and layered onto Lympholite-H, then centrifuged at 2,000 rpm for 25 minutes. Mononuclear cells were collected and washed twice with HBSS, centrifugating at 1,500 rpm for 10 minutes. Finally the pellet was resuspended in Roswell Park Memorial Institute medium (RPMI), containing 5% (v/v) fetal calf serum (FCS) and 100 U/ml P/S. PBMCs were plated for the experiments at a concentration of 4 X 10^5 cells/well in 96 well plates.

2.2.3 Human macrophages

Monocytes were isolated from PBMCs by density gradient centrifugation on iso-osmotic Percoll at 2,000 rpm for 15 minutes. Iso-osmotic Percoll was prepared by mixing 1 volume 1.5M NaCl with 9 volumes of Percoll^{TM}-PLUS. The gradient was then prepared by mixing 1:1 (v/v) iso-osmotic Percoll with 1X PBS/citrate. Monocytes were collected, washed with HBSS and resuspended in RPMI (containing 5% (v/v) FCS and 100 U/ml P/S). Cells were plated in 10 cm dishes at a concentration of 15 X 10^6 cells/dish and macrophage colony-stimulating factor (M-CSF) 100 ng/ml was added. After 4 days, monocytes differentiate to macrophages. Macrophages were harvested and plated at a concentration of 2 X 10^5 cells/well in 96 well plates and left to adhere for 6 hours.
Treatments were done according to each experimental scheme.

2.2.4 CG4 cell line

Central Glia-4 cells (CG4) are a rat line of oligodendrocyte precursor cells able to differentiate into oligodendrocytes or type-2-astrocytes.
Cells differentiate into oligodendrocytes by withdrawal of B104 mitogens (contained in B104 conditioned medium) and growth factors (platelet derived growth factor, PDGF and basic fibroblast growth factor, bFGF). B104-conditioned medium (B104-CM) was obtained from culture of the rat neuroblastoma B104 cell line in DMEM without serum but with addition of N1 supplement.
CG4 and B104 cells were both kindly provided by Professor Yuti Chernajovsky (William Harvey Research Institute in London – WHRI).
CG4 cells were plated in poly-L-ornithine (15 μg/ml) coated culture plates or flasks, and maintained at the precursor stage by culture in growth medium (GM): Dulbecco's modified Eagle's medium (DMEM) containing biotin, bFGF, PDGF, N1 supplement (insulin, transferrin, sodium selenite, putrescine, progesterone) and B104-CM (Table 2.1).
For differentiation, CG4 cells were cultured in differentiation medium (DM): DMEM/F12 medium without growth factors and mitogens (bFGF, PDGF

and B104-CM) supplemented with: progesterone, putrescine, sodium selenite, insulin, transferrin, biotin, thyroxine, glucose (Table 2.2).
CG4 cells were plated at different concentrations, depending on experiments, in GM. After one or two days cells were differentiated and treated according to the experimental scheme. Triplicate samples were analysed for each experimental condition; each sample was obtained by pooling two wells of a 24 well plate.
CG4 cells in the undifferentiated and differentiated stage are shown in Fig. 2.1.

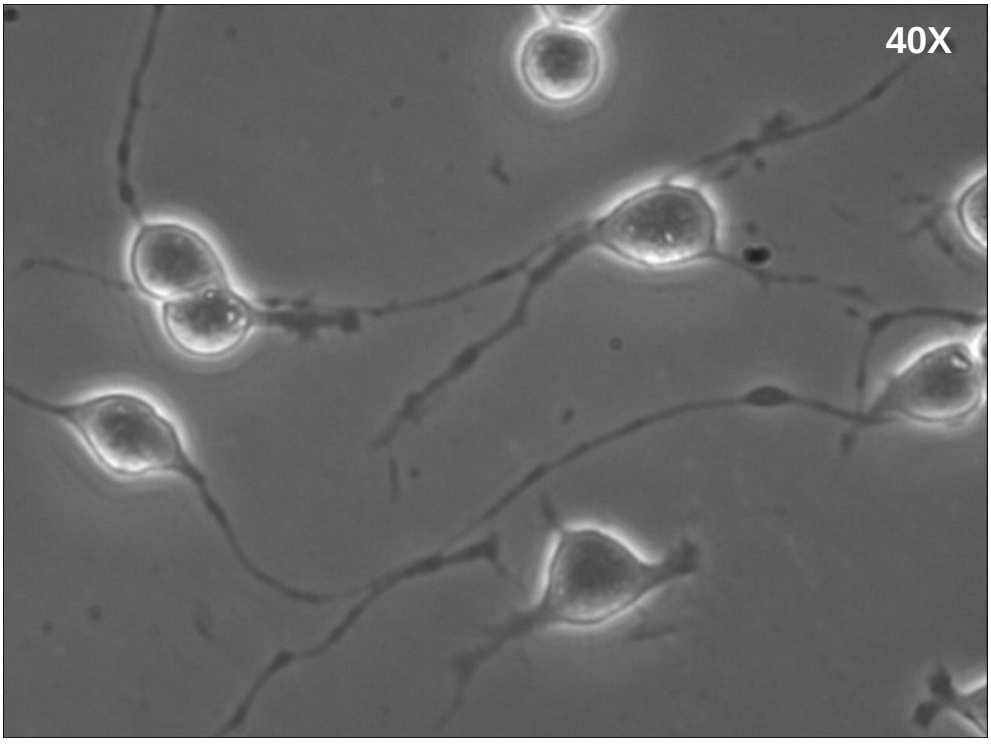
Growth medium (GM)	
COMPONENTS	FINAL CONCENTRATION
B104-conditioned N1 medium	15 ml in 50 ml GM
N1 supplement	-
Biotin	10 ng/ml
bFGF	5 ng/ml
PDGF	1 ng/ml

Table 2.1: Components of the CG4 growth medium.

Differentiation medium (DM)	
COMPONENTS	FINAL CONCENTRATION
Progesterone	3 ng/ml
Putrescine	5 μg/ml
Sodium selenite	4 ng/ml
Insulin	12.5 μg/ml
Apo-Transferrin	50 μg/ml
Biotin	10 ng/ml
Thyroxine	0.4 μg/ml
Glucose	3 g/l
Penicillin	50 U/ml
Glutamine	2 mM
Streptomycin	50 μg/ml
DMEM/F12	-

Table 2.2: Components of the CG4 differentiation medium.

A



B

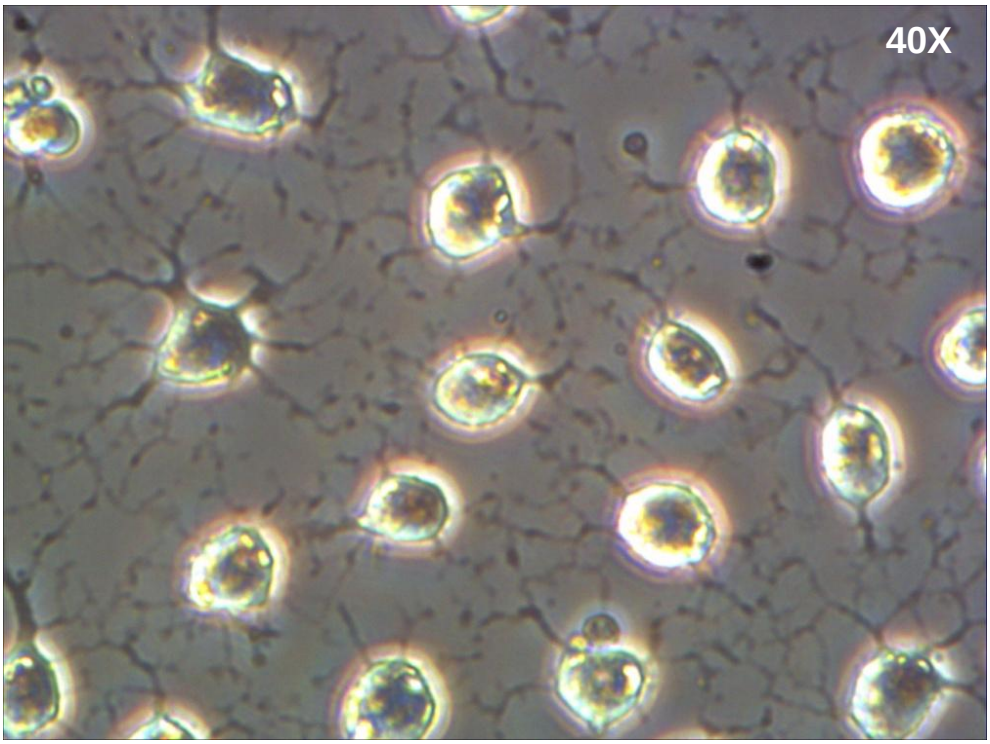


Figure 2.1: CG4 cells in the undifferentiated stage (A) and at 4 days of differentiation (B).

2.2.5 CG4EPOR and CG4 empty vector cells

CG4 cells modified to overexpress EPO receptor (CG4EPOR) were also provided by the Chernajovsky laboratory. These cells were obtained by super-transduction of CG4 cells with EPO receptor gene in a constitutive lentiviral vector. CG4 WT cells were plated at a density of 5 X 10^3 cells/well in 24 well plates in GM. After 4 hours, a lentiviral vector (Demaison et al., 2002), containing EPOR tagged with the V5 epitope, the internal ribosome entry site (IRES, bi-cistronic expression vector) and the enhanced green fluorescent protein (EGFP) reporter, was added at the multiplicity of infection (MOI) of 10 (Fig. 2.2). Culture medium was renewed after overnight culture and the cells were expanded before using them in the experiments.

CG4 empty vector cells (CG4 EGFP) were produced with the same method, by transduction of CG4 WT cells with a lentiviral vector containing only EGFP.

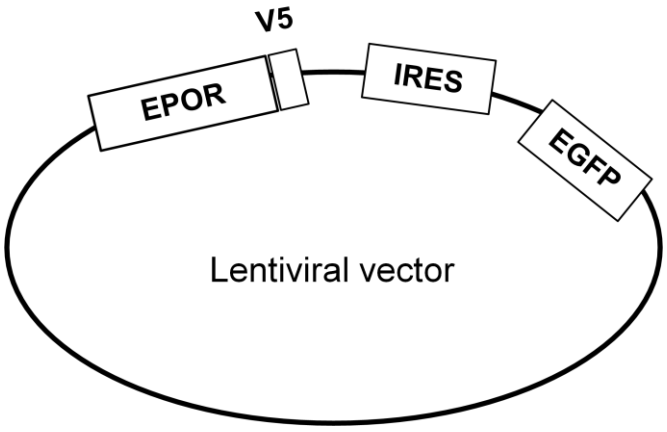


Figure 2.2: Scheme of the bi-cistronic construct used in the lentiviral vector for transfection with EPOR.

2.3 In vivo models

All experimental procedures were performed in accordance with the European Communities Council Directive #86/609 for care of laboratory animals and in agreement with national regulations on animal research in UK.

2.3.1 Induction of precursor cells mobilization in mice

CD1 male mice, 4-6 weeks old, were used. Mice were treated by ip injection with EPO 50 μg/kg (200 μl/30 g mouse of a solution of EPO 7.5 μg/ml in saline) or saline solution for 3 days. Three hours after the last injection mice were anaesthetised (100 μl/30 g mouse of: Ketaset 100 600 μl, Chanazine 2% 300 μl, saline solution 100 μl). The circulating blood was collected with heparinised syringes then mice were sacrificed. In eppendorf tubes 200 μl of whole blood diluted 1:3 with RPMI were added and treated with staphylococcus (kindly provided by Dinarello) (Feezor et al., 2003) or LPS (10 ng/ml, 1 μg/ml or 10 μg/ml). Samples were incubated for 24 hours at 37ºC. Afterwards they were centrifuged at 1200 rpm, 4ºC for 20 minutes. The plasma was collected and frozen at -20ºC to be analysed by ELISA to measure TNFα.

2.3.2 Animal model of demyelination induced by cuprizone

The neurotoxicant Bis(cyclohexanone)oxaldihydrazone or cuprizone (CZ) is a copper-chelator able to induce specific demyelination in the central nervous system with subsequent spontaneous remyelination when mice are fed with normal diet again. CZ was purchased from SIGMA-Aldrich and the diet was prepared by Special Diet Services. C57BL/6J female mice at 7-8 weeks of age were obtained from Charles River. These mice were fed with chow containing 0.2% CZ. Two different time points were considered for demyelination induction: 3 and 5 weeks. Mice were kept on a CZ-containing diet for 3 and 5 weeks and then allowed to recover (removal of CZ from the diet) for an additional one to five days prior to

sacrifice. Controls were fed with normal chow. Animals were treated with EPO 50 μg/Kg every other day. An average of 6 mice per group was used. At the established time points, mice were sacrificed and the brain was removed. The brain region between lambda and bregma, including the corpus callosum, was collected under a dissection microscope. Different gene expressions were studied by qPCR: MOG, MBP, TNFα, CCL2 and the platelet-derived growth factor receptor α (PDGRFα), a marker for oligodendrocyte precursor cells. HPRT1 was used as housekeeping gene.

2.4 Enzyme- linked immune sorbent assay (ELISA)

The ELISA is a specific technique used to quantify cytokines in solutions. This high specificity method uses a sandwich procedure.
A specific monoclonal antibody (mAb), for the cytokine of interest, was coated on the plate with an overnight incubation at 4ºC. The plate was blocked with a reagent diluent (PBS + BSA 1%), by incubation at RT for 1 hour. This step was used to block all unbound sites on the plate. The sample, or the standard, was added into the plate and incubated at RT for 2 hours. A detection polyclonal Ab,(that binds a different epitope on the cytokine) labelled with biotin, was added to the plate for 2 hours at RT. Subsequently, a streptavidin-HRP enzyme was added to the plate for 30 minutes at RT. The streptavidin protein detects biotinilated proteins because it forms high affinity non-covalent bonds with the vitamin biotin. Streptavidin was conjugated with horseradish peroxidise (HRP) to provide enzyme activity for detection, using an appropriate substrate system. Finally, the tetramethylbenzidine (TMB) substrate was added for 20 minutes of incubation at RT. By stopping the reaction with H_2SO_4 2N, there was the development of a coloured product proportional to the amount of cytokine. The absorbance was read at 450 nm with wavelength correction at 540 nm (Microplate reader, Synergy HT, BioTek). Comparing the absorbance of the samples with that of the standard curve, the amount of cytokine present in the samples can be calculated. HuTNF, huIL6 and muTNF Duoset kits from R&D Systems were used.
Anti-huTNF mAb: used at 4 μg/ml for coating. Anti-huTNF Ab: used at 350 ng/ml for detection.
Anti-huIL6 mAb: used at 2 μg/ml for coating. Anti-huIL6 Ab: used at 200 ng/ml for detection.
Anti-muTNF mAb: used at 0.8 μg/ml for coating. Anti-mu TNF Ab: used at 200 ng/ml for detection.

2.5 RNA extraction

Cells were collected in a final volume of 800 μl with TRIzol, able to start the cell lysis.
RNA extraction was started by addition of 160 μl of clorophorm for 800 μl of TRIzol, shaking manually till the solution became homogeneous. Samples were centrifuged at 12,000 g for 15 minutes at 4ºC to obtain a phase separation between the lower phenol-clorophorm phase (red) and the upper aqueous phase (colourless); 200 μl from the colourless upper phase, containing RNA, were transferred in RNAse-free eppendorf tubes without touching the interphase containing DNA and proteins. The same volume of cold isopropanol was added. After shaking, samples were left over-night at 4ºC to allow the RNA to precipitate. The day after, samples were centrifuged at 12,000 g for 30 minutes at 4ºC to precipitate the RNA. All the supernatant was discarded and the pellet washed with 1 ml of ethanol 75% and air-dried. Samples were finally dissolved in 12 μl of RNAse-free water, also by warming up into a thermo block at 60ºC for 10 minutes to help the process. The purity of the RNA was checked by determining the ratio of absorbance at 260 nm to absorbance at 280 nm in a spectrophotometer (NanoDrop ND-1000, Thermo Scientific) (1 O.D. is about 40 μg of RNA/ml) (Fig. 2.3).

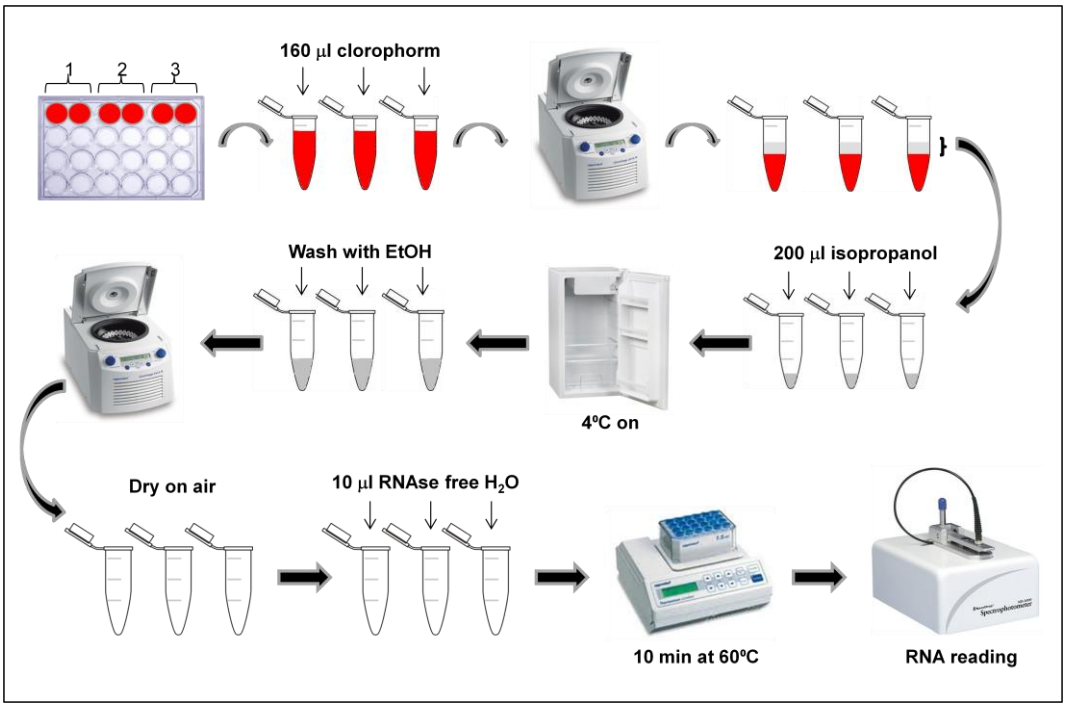


Figure 2.3: Scheme of the RNA extraction process.

2.6 Reverse transcription (RT)

The RT is the process by which single-stranded RNA are retro-transcribed to complementary DNA (cDNA) single-strands.
Total RNA was retro-transcribed to obtain cDNA for the PCR analysis. RT was carried out in 25 μl RT mixture. In a first step, the diluted RNA was incubated with 500 ng random primers and 0.6 mM dNTP for 10 min at 60ºC (TC-3000X). After that, samples were incubated again with RT Buffer, DTT 10 mM, RNAse OUT 40 U and M-MLV-RT 200 U. M-MLV is the Moloney Murine Leukemia Virus Reverse transcriptase that in presence of a primer uses a single-stranded RNA to synthesize the complementary DNA strand. The obtained cDNA can be stored at -20ºC.

2.7 Polymerase chain reaction (PCR)

The PCR is a method to amplify nucleic acid fragments, developed by Kary Mullis in the 1980s. A Taq polymerase, a heat resistant enzyme, is able to synthesize new strands of DNA using a template and primers. The template is the DNA containing the sequence that need to be amplified and the primers are single stranded DNA, complementary to the target sequence, offering the 3'-OH group from where the DNA polymerase can start the synthesis. The PCR starts in this way to generate new copies of the target in an exponential way.
Normal PCR is not quantitative since the results are obtained from the end point, when the reaction arrives to a plateau phase.

2.7.1 Quantitative PCR (qPCR)

A quantitative real time PCR has been used here with the advantage of detecting PCR amplification during the early phase of the reaction, the exponential phase. Real time PCR has been performed using brilliant II QPCR Master Mix, DNA primers and the fluorescent Taqman probe (Applied Biosystem). The brilliant II QPCR Master Mix included: nucleotides, an optimized QPCR buffer, a Taq polymerase and a reference dye (ROX). ROX is present in a low concentration (30 nM) in the mix and its fluorescence should be the same during the PCR reaction, therefore it can be considered as a baseline to which samples are normalised. The Taq polymerase is a SureStart Taq that has high specificity. Taqman probes have a fluorophore usually at the 5' end of the probe and the quencher at the 3' end. When the probe is intact, fluorophore and quencher are close to each other and there is no fluorescence observed from the fluorophore. During the annealing-extension step of the PCR, primers and the Taqman probe hybridize with the target (Fig. 2.4).

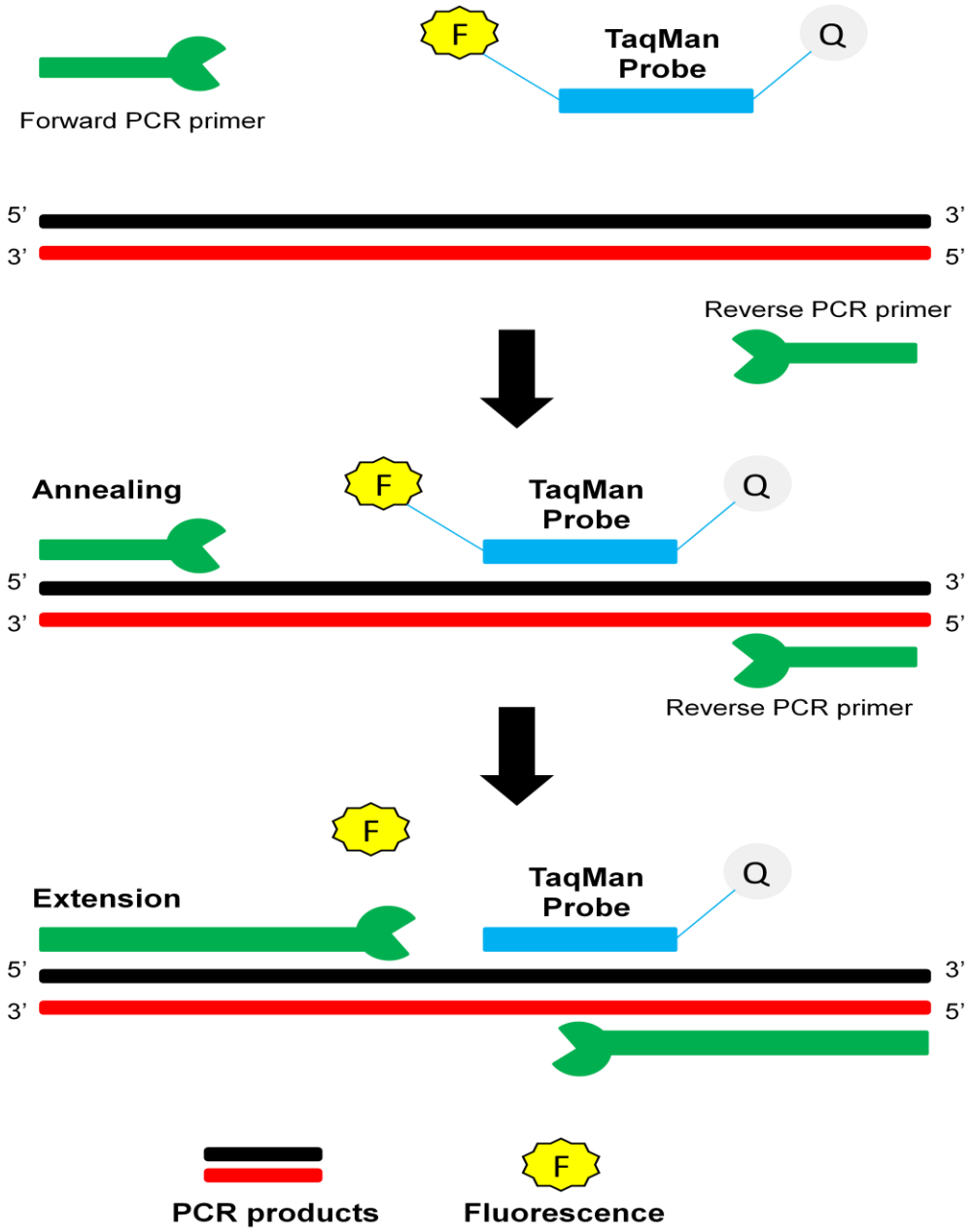


Figure 2.4: Scheme of PCR reaction with the Taqman method.

During the extension step, the Taq polymerase displaces the Taqman probe and cleaves it, since it also has exonuclease activity. Since the fluorophore is separated from the quencher it is possible to detect by real time PCR the fluorescence obtained during each PCR cycle (Fig. 2.4). The step-cycling protocol in the Taqman reaction is:

Cycles nº	Cycle setting	Meaning of the cycle
1 cycle	10 minutes 95ºC	INITIALIZATION STEP: the Taq polymerase is activated
40 cycle	15 seconds at 95ºC	DENATURATION STEP: the cDNA template is melt to obtain two single strands of DNA
	1 minute at 60ºC	ANNEALING-EXTENSION STEP: Taq DNA polymerase extends the DNA from the primers.

A PCR mix is prepared, for each sample, by addition of 12.5 μl of 2X qPCR Master Mix, 1.25 μl of 20X Gene expression assay mix, 9.25 μl of H2O; then 2μl of cDNA are added. The gene expression assays used were: TREM-1, MOG, MBP, TNFα, CCL2, PDGFRα, EPOR, EGR2, mTOR. As housekeeping gene HPRT1 was used. Sequences for qPCR were all of commercial source. All procedures were performed on the MX 3000P Sequence Detection System (Stratagene, Agilent) and the analysis was based on the ΔΔCt Method (Livak and Schmittgen, 2001). The threshold cycle (Ct) is defined as the cycle at which the fluorescence is determined to be statistically significant above background. All quantifications were normalised to an endogenous control, the housekeeping gene.

The analysis of the relative quantification required calculations based on the Ct as follows: 1) ΔCt, the difference between the Ct value of the sample evaluated with the gene of interest and that of the same sample evaluated with the housekeeping gene; 2) $\Delta\Delta$Ct, the difference between the Ct value of the sample and the Ct value of a control sample used as a calibrator; 3) $2^{-\Delta\Delta Ct}$, which yields the final gene expression units or fold induction versus the calibrator, indicated as arbitrary units.

The SYBR-Green is an alternative method to the Taqman PCR that uses a double stranded DNA binding dye to detect PCR products. This qPCR does not require a probe; therefore it is cheaper than the Taqman assay. On the other hand, the SYBR-green assay may detect all double-stranded DNA amplified, including non-specific reaction products. This is a disadvantage, since it may generate false-positive signals, and therefore preliminary experiments need to be done to rule out the presence of non specific products.
This method was used for preliminary experiments to analyse EPO receptor and TREM-1 gene expression. Two μl of cDNA were incubated with:

- SYBR Green PCR Master Mix 2X 12.5 μl
- Primers 5μM each 1.5 μl + 1.5 μl
- H_2O 7.5 μl

The analysis of the relative gene expression was done as described above for the Taqman PCR.

2.8 Cell viability assay

The CellTiter-Blue Cell Viability Assay (CTB) is a fluorescent method to detect cell viability. Living cells are able to convert a redox dye (resazurin) into a fluorescent end product (resorufin), whereas dead cells are not able to do it since they have lost their metabolic capacity.
Cells were cultured in a 96-well plate at different concentrations depending on experiments. The following day, cells were differentiated and treated with EPO. At the established time point 20μl of CTB reagent were added to each well. After 4 hours of incubation the fluorescence was recorded using a fluorescence microplate reader (Synergy HT, BioTek) with excitation 530/25 nm and emission 590/40 nm filters.

2.9 Cell cloning by limiting dilution

Cell cloning can be done by limiting dilution. Cells were plated at a low concentration in order to have a very high probability to have a single cell in a well, therefore a cell line derived by a single cell. In this procedure 100 cells were distributed between 288 wells (3 96 well plates), one cell per 3 wells (0.3 cell/well). Approximately 25 colonies were expected after two weeks.

Cells were diluted firstly at a concentration of 1 X 10^5 cells/ml in 1 ml. Then three 10-fold serial dilutions were done. By addition of 29 ml to the last tube, containing 100 cells in 1 ml, the final concentration obtained was 100 cells in 30 ml. Since 100 μl of solution were dispensed in each well the final concentration was 0.3 cell/well (Fig. 2.5). After 3-4 days half of the medium was changed and new growth medium added with the double amount of bFGF and PDGF.

Wells containing more than one cell (as seen by the presence of more than one colony after about 2 weeks) were excluded from the experiment. After cell expansion, part of the clones was frozen and part was harvested using TRIzol reagent for RNA extraction, reverse transcription and EPO receptor detection by qPCR. Some of these clones were also treated with EPO 80 ng/ml throughout the 6 days of differentiation. MOG and MBP gene expressions were analyzed by qPCR.

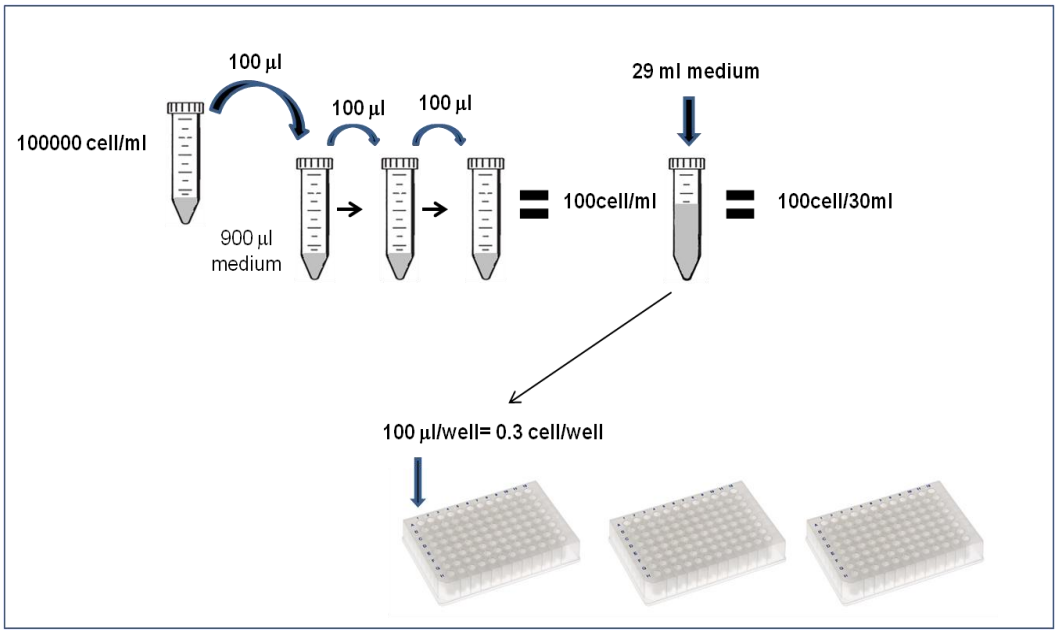


Figure 2.5: Scheme of cloning by limiting dilutions.

2.10 Gene silencing

Gene silencing is a technique used to down-regulate the expression of specific genes by introducing a double-stranded RNA complementary to the target RNA of interest. To this aim a small interfering RNA (siRNA), around 21 base pairs long, was used. The transfection is the critical step of this method. To silence the gene expression we used Silencer Select Pre designed siRNA for EGR2 and a negative control siRNA.
The transfection was done with two procedures: reverse and forward. In the reverse method cells were plated and transfected at the same time, in the forward one, cell were plated and the day after differentiated with DM and transfected: 35 X 10^3 cells/ml were plated in GM without P/S.
For each well to be transfected, the preparation was:

a) 1,8 μl siRNA 30 nM in 50 μl OPTIMEM.
b) 1 μl lipofectamine RNAiMAX in 50 μl OPTIMEM.
c) Mix gently the two preparations and incubate for 10 minutes at RT.
d) Add 100 μl to each well.

Gene silencing was confirmed by qPCR for the gene of interest.

2.11 Protein analysis by Western Blotting (WB)

CG4 WT and CG4EPOR cells were plated 1 X 10^5 cells/10 cm plate in Petri dishes in GM. When sub-confluent, cells were differentiated with DM for 3, 6 or 9 days, changing the medium every day after day 3 of differentiation. The culture medium was renewed 2 hours before lysing the cells. Cells were collected and centrifuged at 4ºC, 500g for 3 minutes. Supernatants were removed and pellets lysed in 50 μl of lysis buffer on ice for 20 minutes with occasional vortexing. The lysis buffer recipe is:

Compounds	Final concentration
Tris HCl pH 7.4	10 mM
NaCl	100 mM
Na4EDTA	1 mM
EGTA	1 mM
NaF	1 mM
SDS	0.1%
Sodium deoxycholate	0.5%
Triton X100	1%
Glycerol	10%
$Na_4P_2O_7$	20 mM
PMSF	1 mM
Protease inhibitor cocktail	-
$NaVO_4$	2 mM

Supernatants were cleared by centrifugation in microfuge for 10 minutes at 4 ºC, full speed and frozen. Protein concentration was measured by using BCA kit (Pierce). 100 μg of protein were loaded per lane on Tris/SDS 10% polyacrilamide gel without β-mercaptoethanol and subjected to electrophoresis. This is a method to separate proteins according to their different molecular weight. The separation is determined by the relative size of the pores formed within the gel. Separated proteins were blotted using Trans-Blot Turbo Blotting System (Biorad) onto a Polyvinylidene fluoride (PVDF) membrane. The membrane was blocked, to prevent non

specific background, with 2% casein in PBS. Antibodies were diluted in PBS + 2% casein + 0.1% Tween 20.

1. Immunoblotting with anti-MOG Z12 1:50; goat-anti-mouse IgG-HRP (Santa Cruz #3697) 0.4 mg/ml; exposure 50 minutes by enhanced chemiluminescence (ECL).
2. Immunoblotting with mouse anti-β-actin (Sigma, clone AC-15, #A54541) 1:20,000, goat-anti-mouse IgG-HRP (Santa Cruz #3697) 0.2 mg/ml; exposure 5 seconds by ECL.

2.12 Primary oligodendrocyte cells

Primary mixed glial cultures from P0-P3 neonatal rats were generated as described by McCarthy et al. (McCarthy and de Vellis, 1980); with minor modifications. Neonatal pups were euthanized according to Schedule 1 regulations from the Home Office Animal Procedures Committee UK (APC). Neonatal rat cortices were stripped from overlying meninges, white matter was removed and hemispheres were separated. Cortical tissue was then digested for 30 minutes at 37°C in a papain (Sigma) solution containing DNase (Sigma) and L-cysteine (Sigma). A single cell solution was obtained after additional mechanical homogenization. Mixed glial cells from 2 brains were plated onto one poly-L-lysine (Sigma) coated T75 flask. Mixed glial cultures were cultured for 10 days at 7.5% CO2 in a high glucose DMEM (Sigma) medium containing 10% FBS (Sigma) and L-glutamine (Sigma). Medium was changed every 3 days. After 10 days of cultivation, mixed glial cultures consisted of a basal layer containing fibroblasts and astrocytes and a top layer containing OPCs and microglia. Cultivation flasks were agitated for one hour on a rotary shaker (260rpm, 37°C) to remove microglia from the top layer of the cultures. After changing the medium, flasks were then gently shaken by hand to obtain a cell suspension primarily containing OPCs. Remaining microglia and dead cells were removed by performing a 15 minutes differential adhesion step on non tissue culture coated petri dishes. Finally, OPCs that remained in suspension during the differential adhesion step were collected and plated onto poly-L-lysine coated 6-well plates. The purity of cells was analyzed by quantification of immunocytochemistry against the oligodendrocyte precursor cell marker O4, the astrocyte marker GFAP or the microglial marker Lectin.

OPC cultures were cultivated in chemically-defined Satos medium (DMEM + L-glutamine, insulin, holo-transferrin, bovine serum albumine, progesterone, putrescine, sodium selenite, T3, T4) that was either supplemented with 0.5% FCS (PAA) to induce differentiation or PDGF-AA (Preprotech, 10ng/ml) and FGF (Preprotech, 10ng/ml) to maintain a proliferative precursor state.

At the end of the experiment, OPCs were lysed, scraped and total RNA was extracted using the RNAeasy Mini kit (Qiagen). Total RNA was retrotranscribed to obtain cDNA using a First strand RT kit (Roche). Primers (Sigma) for SYBR Green real time PCR were designed. Real-time PCR was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). Relative quantification was performed using the $\Delta\Delta$CT method, as described above in 2.7.1, using beta-actin as endogenous control gene (kind donation of Kathryn Blair, MRC Centre for Stem Cell Research, University of Cambridge, UK).

Chapter 3: EPO in inflammation

Several studies demonstrated an anti-inflammatory effect of EPO *in vivo* by reduction of inflammatory cytokines, for instance in multiple sclerosis and cerebral malaria (Savino et al., 2006; Kaiser et al., 2006). However, as discussed in the introduction, the demonstration of a direct anti-inflammatory effect of EPO *in vitro* is controversial and it seems to be a consequence of the anti-apoptotic one.

The aim of this chapter was to better investigate whether the effect of EPO observed *in vivo* was indirect, by reduction of apoptotic death, or due to a direct antagonism of inflammation. First of all, the lack of EPO effect on LPS-induced cytokines was investigated to confirm previous results. Subsequently, the hypothesis studied in this chapter was that EPO could decrease inflammatory cytokines induced by other pathways rather than LPS. In order to investigate this idea, several inductors were considered, trying to reproduce the mechanisms activated during diseases. The main hypothesis was the involvement of alarmins, since they induce inflammation in several diseases and CNS injuries. HMGB1 and the necrotic cell lysate (NCL) can induce cytokines *in vitro* (Andersson et al., 2000; El Mezayen et al., 2007) and ATP plus a costimulus can activate the inflammasome, involved in mediation of inflammation (Franchi et al., 2009). In addition, it was found in our laboratory that in an *in vivo* model of stroke, induced by middle cerebral artery occlusion (MCAO), EPO decreased the expression of the pro-inflammatory triggering receptor expressed on myeloid cells 1 (TREM-1). Thereby, we also looked at the effect of EPO on TREM-1 *in vitro*.

Several cell types have been used as *in vitro* models. MonoMac6 cells (MM6), a line of monocytes, were used in these experiments, since monocytes are mainly involved in defence. Monocytes migrate in tissues in response to an inflammatory insult, then differentiate to macrophages and start the immune response. Moreover, before starting all the experiments, the expression of EPO receptor in MM6 was checked. The effect of EPO

was also studied in some experiments in primary peripheral blood mononuclear cells (PBMCs) and macrophages, also to clarify that the lack of EPO effect was not correlated to the use of a cell line. In these cells the expression of EPO receptor was already well documented.
Finally, an indirect anti-inflammatory pathway was also considered, starting from the evidence that EPO induces mobilization of endothelial precursor cells. The hypothesis was that these stem/precursors could have a role in mediation of the anti-inflammatory effect of EPO. To this aim, an *in vivo* study using mice was established.

3.1 EPO does not have any effect on LPS-induced IL6

The effect of EPO on LPS-induced IL6 in PBMCs was here investigated to confirm the findings by Villa which states that there was no effect of EPO on TNFα secretion induced by LPS in PBMCs (for PBMCs preparation see section 2.2.2, pag. 40) (Villa et al., 2003).
Heparinised blood was collected from two donors and PBMCs were isolated as described in the methods chapter. Cells were plated at a concentration of 4 X 10^5 cells/well in 96 well plates and stimulated with LPS 0.1 ng/ml for 24 hours with or without EPO 80 ng/ml, added half an hour before LPS. After the stimulation time, supernatants were collected and analysed for IL6 by ELISA. IL6 was undetectable in control samples (<10 pg/ml) and induced at high levels by incubation for 24 hours with LPS (DONOR 1: 6572 ± 243.3 pg/ml; DONOR 2: 12073 ± 1375 pg/ml).
However, there was no effect of EPO on the secretion of this inflammatory cytokine (DONOR 1: 5980 ± 363 pg/ml LPS+EPO *vs* 6572 ± 243.3 pg/ml LPS; DONOR 2: 11899 ± 565 pg/ml LPS+EPO *vs* 12073 ± 1375 pg/ml LPS) (Fig. 3.1).
Therefore, it was concluded that EPO does not have any anti-inflammatory effect on cytokines induced *in vitro* by LPS. This was the starting point for investigating the effect of EPO on other pathways mediating inflammation.

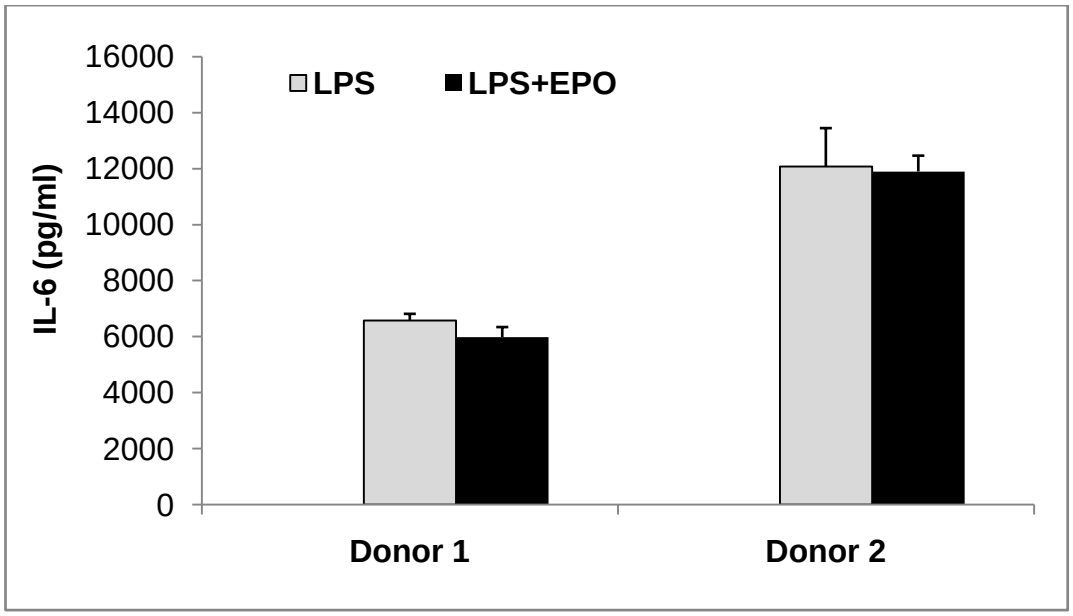


Figure 3.1: EPO does not have any effect on IL6 induced by LPS in PBMCs. Blood was taken from 2 healthy donors in heparinized tubes and PBMCs were isolated. Cells were plated at a concentration of 4 X 10^5 cells/well in 96 well plates. Cells were stimulated with LPS 0.1 ng/ml with or without a pre-treatment with EPO 80 ng/ml, half an hour before the stimulation. After 24 hours supernatants were collected for IL6 detection by ELISA. Results are the average ± SD of three samples analysed in duplicate. EPO did not modulate IL6 induced in PBMCs by LPS. IL6 expression in CTRL cells was less than 10 pg/ml, hence not represented in the histogram.

3.2 Effect of EPO on cytokines induced by alarmins

3.2.1 Effect of EPO on HMGB1-induced cytokines in MonoMac-6 cells

HMGB1 is a protein triggering inflammation, released by necrotic cells (Scaffidi et al., 2002), but also secreted by activated monocytes and macrophages (Klune et al., 2008). HMGB1 is overexpressed in several pathological conditions like autoimmune diseases (rheumatoid arthritis, lupus erythematosus and multiple sclerosis), stroke, cancer, etc... (Andersson et al., 2008; Ha et al.). Because HMGB1 treatment stimulates pro-inflammatory cytokine synthesis in PBMCs *in vitro* (Andersson et al., 2000; Hreggvidsdottir et al., 2009), the aim of this experiment was to study the anti-inflammatory effect of EPO in a model that could mimic pathways activated by diseases.

MM6 cells, a line of monocytes, were used as an *in vitro* model. Before using MM6 cells to study the EPO effect on inflammation, the expression of EPO receptor was checked, to ensure they could be responsive to EPO. MM6 cells were analysed by Sybr Green PCR for EPO receptor expression and compared with PBMCs, in which the expression of EPO receptor had been already demonstrated (Lisowska et al., 2010). The basal expression of EPO receptor was similar in the two groups without significant difference (for similar housekeeping expression the Ct value was 16.5 in MM6 cells compared to a Ct of 18 in PBMCs).

MM6 cells were plated at a concentration of 4×10^5 cells/well in 24 well plates. One hour later, they were treated with a concentration of 1 μg/ml of HMGB1. The dose of 1 μg/ml, was reported (Andersson et al., 2000; Yang et al., 2010) to induce high levels of IL6 without using any co-stimulus. HMGB1 was reported to induce IL6 at short time points and at 24 hours (Andersson et al., 2000). To find the best time for IL6 induction, supernatants were collected after 10 and 24 hours and analyzed by ELISA for IL6 and TNFα expression.

TNFα was undetectable in supernatants of control and HMGB1-stimulated MM6 cells. A good induction of IL6 was observed at both the time points (arbitrary units: 79.9 ± 1.8 pg/ml by HMGB1 at 10 hours *vs* 105.2 ± 10.2 pg/ml by HMGB1 at 24 hours; $P < 0.01$) (Fig. 3.2). IL6 secretion was significantly higher at 24 hours rather than at 10 hours and consequently this time point was chosen for the following experiments.

Once the optimal induction time was found, a dose-response experiment with EPO was set up, treating MM6 cells with different concentrations of EPO half an hour before HMGB1 treatment. The usual dose of EPO used *in vitro* to protect from apoptosis is 80 ng/ml. The EPO concentration necessary to mediate the anti-inflammatory effect was completely unknown, therefore several concentrations of EPO were studied. Supernatants were collected after 24 hours and analysed by ELISA for IL6.

As shown in Fig. 3.3, there was a good induction of IL6 by HMGB1 compared to the control (98.6 ± 11.8 pg/ml in HMGB1 group *vs* 1.83 ± 0.45 pg/ml in the control). Unfortunately, no effect of EPO on HMGB1-induced IL6 was found at any of the doses used. At the dose of 1 ng/ml, there was a weak effect of EPO in decreasing IL6 (98.6 ± 11.8 pg/ml by HMGB1 alone *vs* 81.9 ± 8.2 pg/ml by HMGB1+EPO 1 ng/ml; $P< 0.05$), but since the fold change was <1.5 this effect was not considered significant (Fig. 3.3).

In conclusion, HMGB1 induced IL6 but not TNF in MM6 cells at the dose of 1 μg/ml and at the time points considered of 10 and 24 hours. However, EPO did not decrease IL6 expression at any dose used (range between 0.1 ng/ml and 1000 ng/ml) after 24 hours.

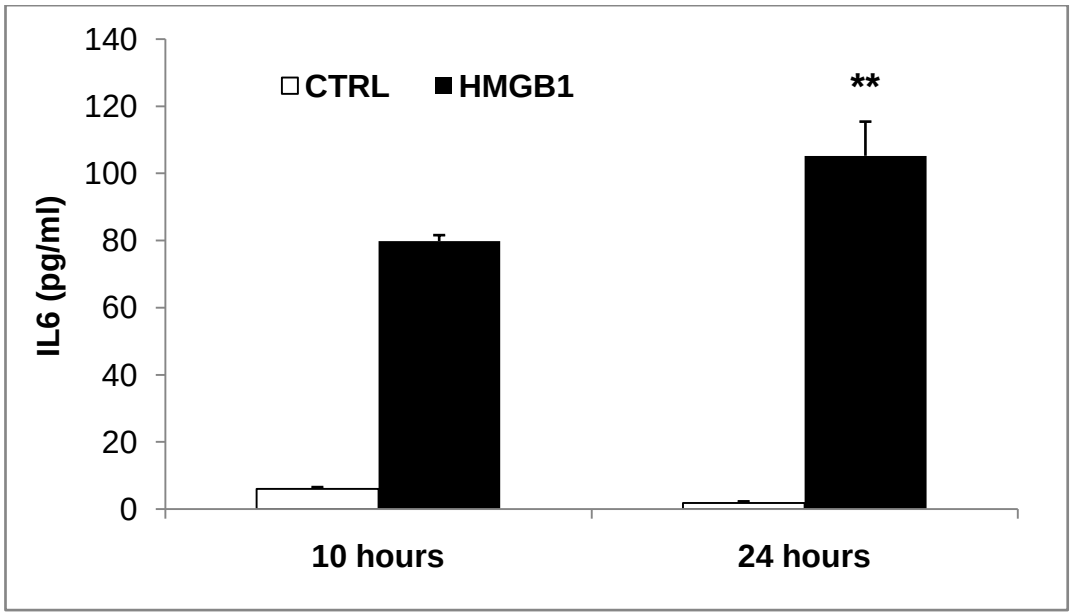


Figure 3.2: HMGB1 induces IL6 at 24 hours better than at 10 hours. MM6 cells were plated at a concentration of 4 X 10^5 cells/well in 24 well plates and one hour later they were treated with HMGB1 1 μg/ml. Supernatants were collected after 10 hours and 24 hours to analyse IL6 induction by ELISA. Results are the average of three samples analysed in duplicate ± SD. **P<0.01 *vs* HMGB1 10 hours.

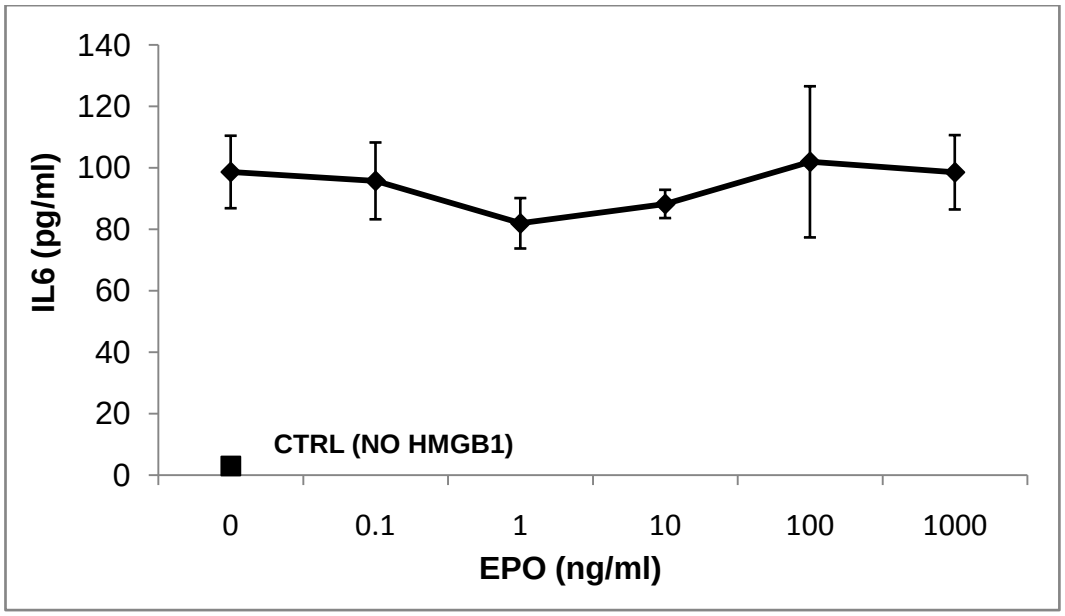


Figure 3.3: EPO does not decrease IL6 induced by HMGB1. MM6 cells were plated at a concentration of 4 X 10^5 cells/well in 24 well plates and after one hour they were treated with the indicated concentrations of EPO. Half an hour later, cells were treated with HMGB1 1 μg/ml. 24 hours later supernatants were collected and analysed by ELISA for IL6 expression. Results represent mean ± SD of 6 samples from two different experiments analysed together.

3.2.2 Effect of EPO on HMGB1 induced IL6 in PBMC

The experiment described in section 3.2.1 was repeated in human PBMCs. This was done to assess whether the effect of EPO could be lost in the cell line, and looking at primary cells might be a better way to investigate the role of EPO on the modulation of cytokines induced by HMGB1.

Blood from two donors was collected and PBMC were isolated as described in the methods chapter. 4 X 10^5 cells/well were plated in 96 well plates and stimulated with HMGB1 (1 μg/ml) to induce cytokines production. Cells were pre-treated half an hour before with or without EPO 80 ng/ml. After 24 hours, supernatants were collected and analysed by ELISA for IL6. IL6 was undetectable in unstimulated cells and significantly induced by HMGB1 (DONOR 1: 29958.6 ± 314 pg/ml; DONOR 2: 33590.6 ± 5020 pg/ml) (Fig. 3.4). However, no effect of EPO was observed (DONOR 1: 24228.9 ± 8172 EPO *vs* 29958.6 ± 314 NO EPO; DONOR 2: 29872.3 ± 1797 EPO *vs* 33590.6 ± 5020 NO EPO) (Fig. 3.4).

In conclusion, even in primary cells, EPO did not have any anti-inflammatory effect *in vitro*, at least in terms of modulation of IL6 induced by HMGB1.

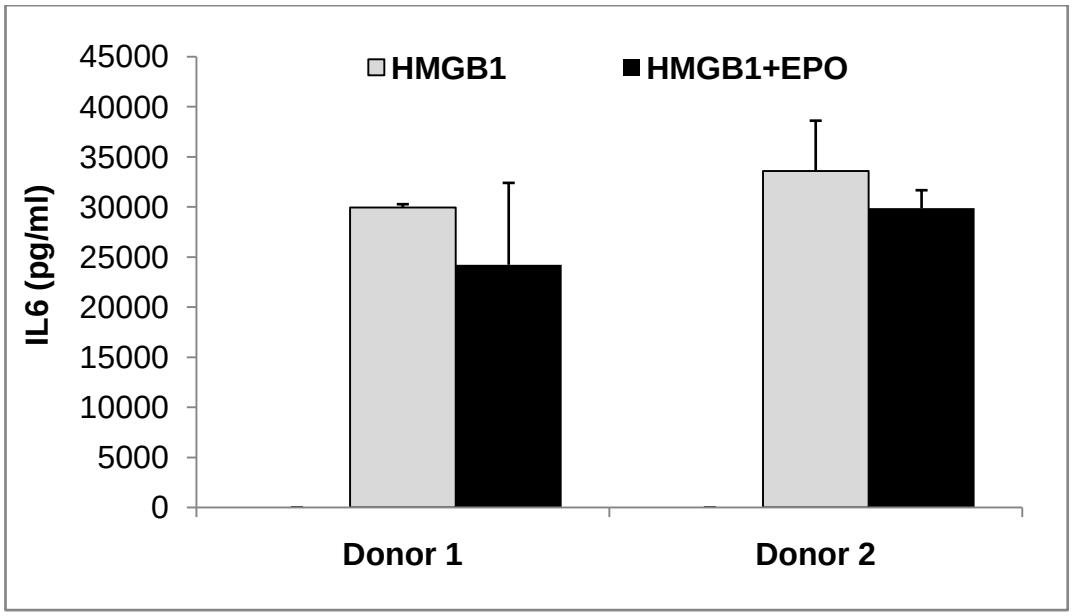


Figure 3.4: No effect of EPO on IL6 induced by HMGB1. Blood from two donors was collected and PBMC were isolated. 4 X 10^5 cells/well were plated in 96 well plates and stimulated with HMGB1 1 μg/ml to induce cytokine production. Cells were pre-treated half an hour before with or without EPO 80 ng/ml. After 24 hours supernatants were collected and analysed by ELISA for IL6 and TNFα detection. Results are the average of three samples analysed in duplicate ± SD from each donor. IL6 expression in CTRL cells was less than 10 pg/ml, hence not represented in the histogram.

3.2.3 EPO effect on necrotic cell lysate (NCL)-induced IL6

HMGB1 is just one of the several danger signals released by necrotic cells.

The aim of this experiment was to investigate whether EPO could modulate cytokine expression in a model of inflammation induced by NCL, possibly by targeting an unknown molecule released by necrotic cells. The idea came from the result obtained in a line of human monocytes, showing that NCL induces inflammatory cytokines (El Mezayen et al., 2007).

According to El Mezayen, NCL was obtained by 6 cycles of freezing and thawing from 3 X 10^5 MM6 cells in 3 ml. After that, cells were centrifuged at 13,500 rpm for 20 minutes at 4ºC. This NCL was subsequently used to treat MM6 cells, plated at a concentration of 8 X 10^4 cells/well in a 96 well plates. Stimulation with NCL in different proportion in the medium, 12%, 25% and 50%, was undertaken for 24 hours to find the optimal amount of NCL requested to induce inflammation.

Cells were treated at the same time with NCL alone or in association with LPS (0.1 and 0.01 ng/ml) as a costimulus. Supernatants were collected and analysed by ELISA for IL6.

NCL alone significantly induced IL6 (11.5 ± 0.1 pg/ml NCL 50% *vs* 1.1 ± 0.2 no NCL; $P < 0.01$) or synergized with LPS 0.1 ng/ml for a higher effect (49.3 ± 2.2 pg/ml NCL 50%+LPS 0.1 ng/ml *vs* 26.2 ± 3.4 pg/ml LPS 0.1 ng/ml alone) (Fig. 3.5). LPS 0.01 ng/ml was too low as dose to have a synergism with NCL (11.7 ± 1.6 LPS 0.01+NCL 50% vs 11.5 ± 0.1 NCL 50% alone) (Fig. 3.5). For this reason, the induction with NCL alone or in association with LPS 0.1 ng/ml was chosen for further experiments.

Following this first result, an EPO treatment was performed, at the dose of 80 ng/ml half an hour before the use of NCL or NCL+LPS, to see its effect in this model of inflammation.

EPO had a really small decreasing effect on NCL-induced IL6 (19.8 ± 1.7 pg/ml NO EPO vs 15.9 ± 1 pg/ml EPO; $P <0.05$). Although the result was significant, because the fold change was <1.5 this effect was not considered relevant (Fig. 3.6).

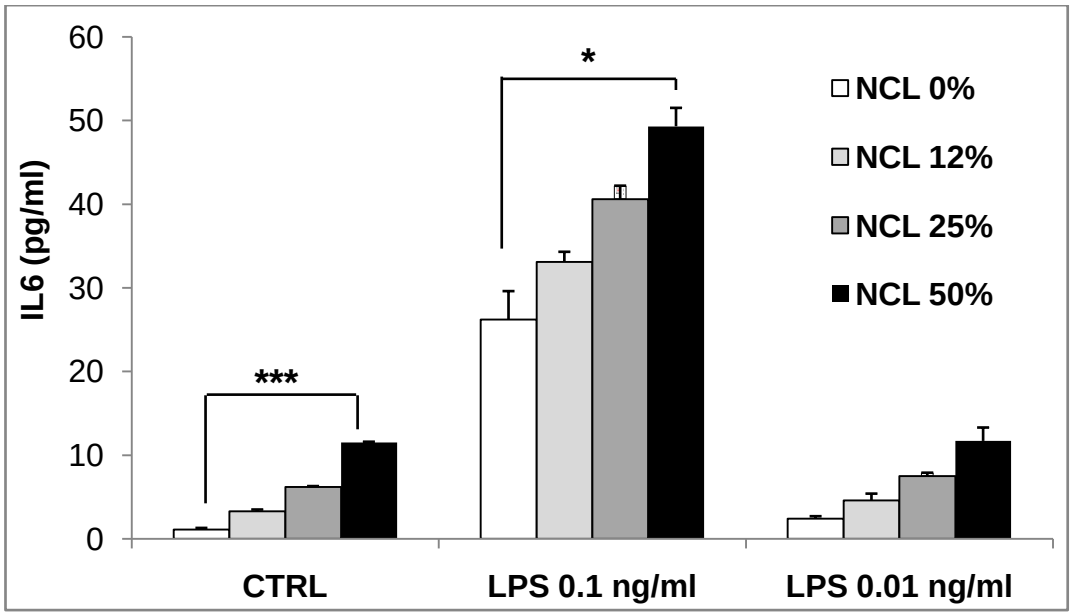


Figure 3.5: Necrotic cell lysate used in a ratio of 50% induces IL6 and synergises with LPS 0.1 ng/ml. MM6 cells were plated at a concentration of 8 X 10^4 cells/well in 96 well plates. Cells were treated with NCL in different proportions in the medium (12%, 25% and 50%) plus LPS in two concentrations (0.1 ng/ml or 0.01 ng/ml). After 24 hours supernatants were collected and analysed by ELISA for IL6 expression. Results are the mean ± SD of triplicates analysed in duplicate. ***P<0.001, *P<0.05 by Student's *t* test.

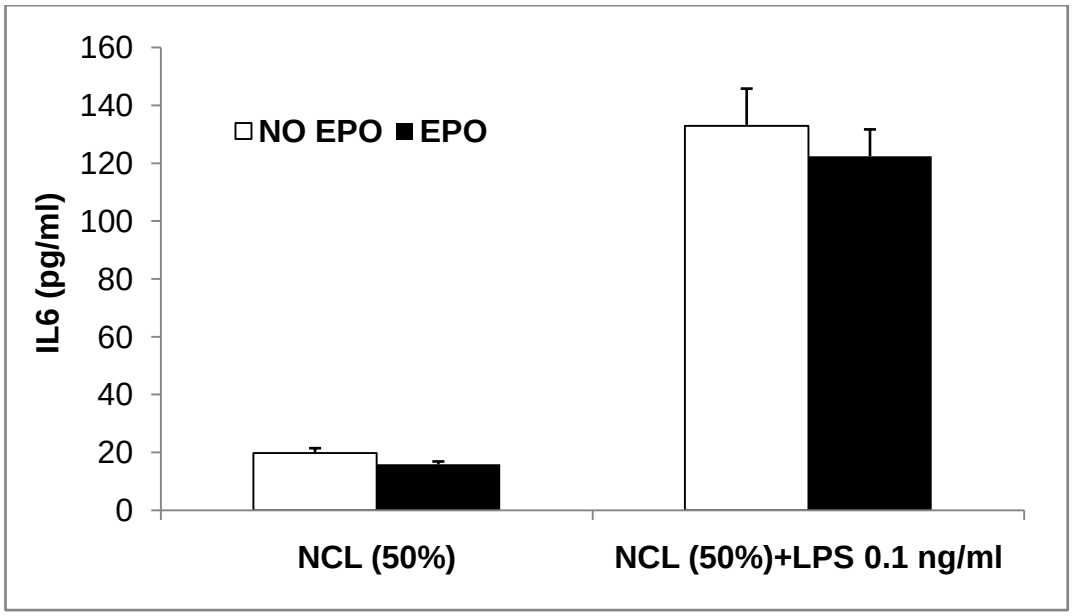


Figure 3.6: EPO does not inhibit IL6 induced by necrotic cell lysate alone or in association with LPS. MM6 cells were plated at a concentration of 8 X 10^4 cells/well. Cells were treated with EPO 80 ng/ml and, half an hour later, with NCL 50% in the medium plus LPS at the concentration of 0.1 ng/ml. After 24 hours supernatants were collected and analysed by ELISA for IL6 expression. Results are the mean ± SD of triplicates analysed in duplicate.

In addition, this effect of EPO was not detected in the group NCL 50% plus LPS 0.1 ng/ml (132.9 ± 12.9 NO EPO vs 122.4 ± 9.3 EPO). In conclusion, no anti-inflammatory effects of EPO were found *in vitro* in this inflammation model in which IL6 was induced by NCL.

3.2.4 Effect of EPO on ATP-induced IL-1β

Adenosine triphosphate (ATP) is considered as another endogenous danger signal. In fact, extracellular ATP is a potent stimulus for the inflammasome via the purinergic receptor P2X (P2X7R), which is a cell surface receptor for ATP in macrophages and other immune cells. The inflammasome is a complex involved in mediation of inflammation. It controls the activation of the enzyme caspase-1 by activating the pro-caspase-1 and subsequently regulates the maturation of IL-1β from the pro-IL1β, induced by some priming stimuli like LPS or TNFα (Franchi et al., 2009). The aim of this experiment was to activate the inflammasome by ATP plus LPS stimulation, to study whether EPO might specifically inhibit that activation.

MM6 cells were plated at a density of 2.5 X 10^5 cells/well in 24 well plates and stimulated with LPS 40 or 100 ng/ml for 6 hours. ATP stimulation was done at 2 mM or 5 mM for the last 60 minutes. IL-1β was measured by ELISA in the supernatants. LPS-primed MM6 cells secreted IL-1β only when further stimulated with ATP. LPS+ATP at the lower doses (LPS 40 ng/ml and ATP 2 mM) induced a good level of IL-1β secretion, that was only slightly increased at the higher doses (LPS 100 ng/ml and ATP 5mM) (Fig. 3.7). For this reason, the lowest doses necessary to induce IL-1β secretion were chosen (LPS 40 ng/ml and ATP 2mM). A second experiment was carried out to investigate the effect of EPO on IL-1β induced through this pathway. MM6 cells were plated again at a concentration of 2.5 X 10^5 cells/well in 24 well plates and treated with EPO 80 ng/ml with two different schemes: before LPS or before ATP. Stimulation with LPS 40 ng/ml was done for 6 hours and with ATP 2mM for the last 60 minutes. Supernatants were collected and analysed by ELISA

for IL-1β. A good secretion of IL-1β was observed by LPS plus ATP as reported in the previous experiment, but EPO did not show any anti-inflammatory effect neither administered before LPS nor before ATP treatment (Fig. 3.8).

In conclusion, EPO did not show an anti-inflammatory effect in any of the models of induction of inflammation by danger signals.

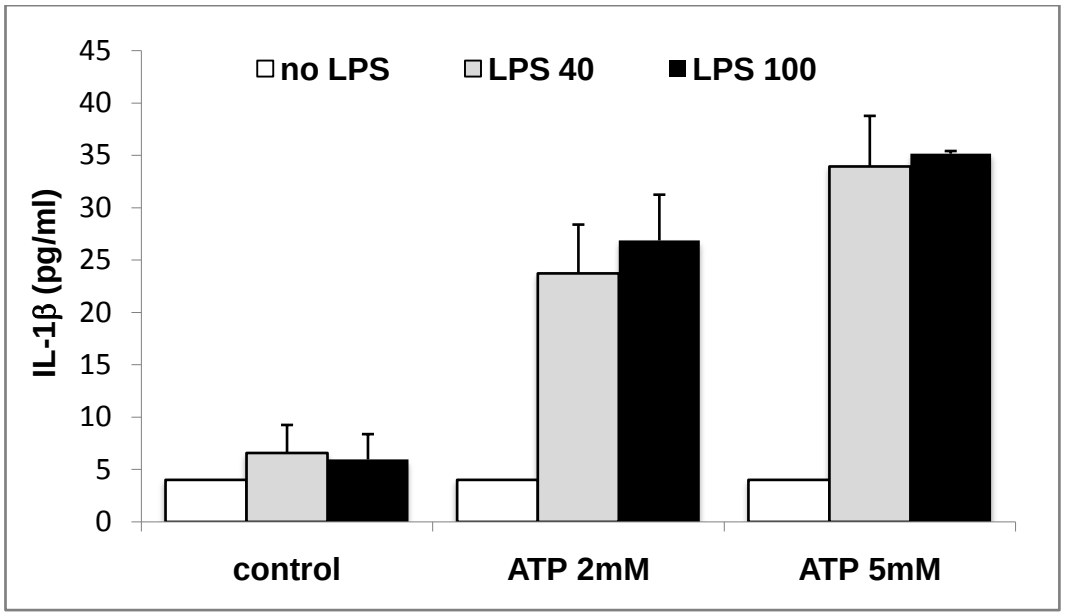


Figure 3.7: Induction of IL-1β secretion in MM6 cells by LPS+ATP. MM6 cells were plated at a density of 2.5 X 10^5 cells/ml in 24 well plates and stimulated with LPS 40 or 100 ng/ml for 6 hours. ATP stimulation was done at 2 mM or 5 mM for 1 hour. IL-1β was measured by ELISA in the supernatants. Results are the mean ± SD of three samples analysed in duplicate.

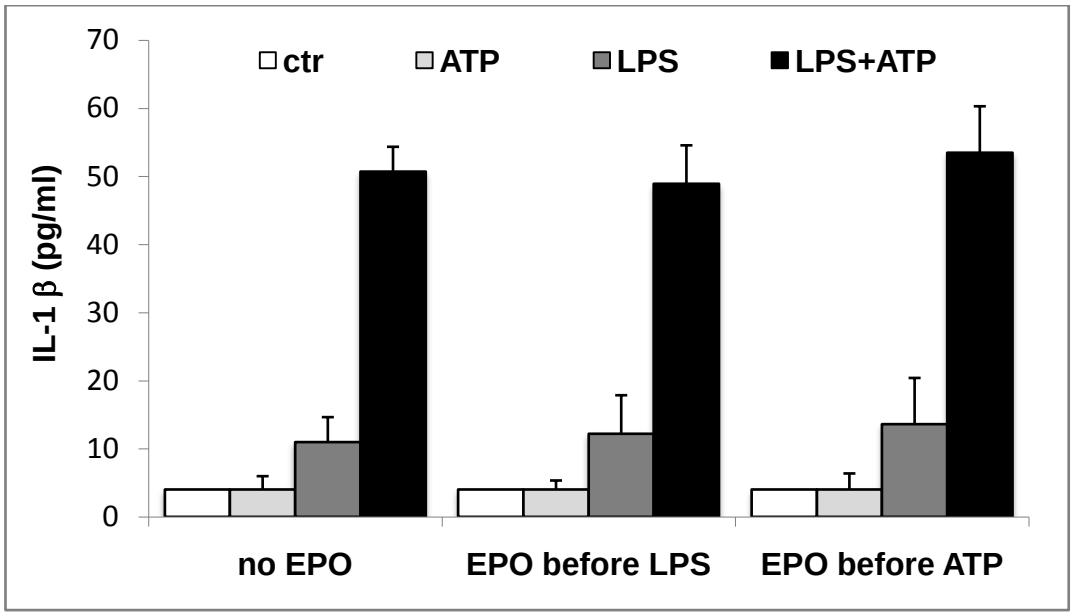


Figure 3.8: EPO does not have any effect on ATP-induced secretion of IL-1β in MM6 cells. MM6 cells were plated at a concentration of 2.5 X 10^5 cells/ml in 24 well plates and treated with EPO 80 ng/ml with two different schemes: before LPS or before ATP treatment. The dose of LPS used was 40 ng/ml for 6 hours and ATP was 2mM for 1 hour. Supernatants were collected and analysed by ELISA for IL-1β. No effect of EPO was seen in any scheme of treatment. Results are mean ± SD of three samples analysed in duplicate.

3.3 Effect of EPO on TREM-1 modulation

The triggering receptor expressed on myeloid cells 1 (TREM-1) is a pro-inflammatory receptor, expressed in neutrophils and monocytes, involved in the amplification of inflammation in ischemic and autoimmune diseases (Bouchon et al., 2000). In previous experiments in our laboratory, it was shown that EPO decreased TREM-1 expression in the ischemic cortex in a rat model of stroke, observed by microarrays studies and confirmed by qPCR. The hypothesis was that EPO could have an anti-inflammatory activity by inhibition of TREM-1. In the course of preliminary experiments carried out in the laboratory, aimed at finding stimuli to induce TREM-1 expression *in vitro*, we had seen that ATP alone was able to induce TREM-1 in MM6 cells at 3 hours.

MM6 cells were plated at a concentration of 25 X 10^4 cells/well in 24 well plates and treated with ATP 4 mM for 3 hours. Cells were collected using TRIzol reagent and the RNA was extracted. Following the reverse transcription, samples were analyzed by Sybr Green qPCR for TREM-1 gene expression, using β-actin as housekeeping gene. ATP alone induced TREM-1 gene expression of about 2 folds (Arbitrary units: 1.7 ± 0.04 ATP *vs* 0.8 ± 0.2 CTRL; $P<0.01$ by Student's *t* test) (Fig. 3.9).

In addition, TREM-1 expression induced by ATP in hypoxic conditions was evaluated to try to increase the effect of ATP alone and the effect of EPO was studied. MM6 cells were treated with ATP 4 mM for 3 hours in normoxic or hypoxic conditions. EPO treatment was done half an hour before ATP stimulation and hypoxia. Cells were collected, 3 hours later, with TRIzol and the RNA was extracted. After reverse transcription, samples were analysed by Taqman qPCR for TREM-1, as the Taqman method is more accurate than the Sybr Green method. There was no difference between TREM-1 induced by ATP in normoxic and in hypoxic conditions (5.4 ± 0.9 hypoxia *vs* 4.2 ± 0.1 normoxia). Unfortunately, no effect of EPO was detected (Normoxia: 5.4 ± 0.6 ATP+EPO *vs* 4.2 ± 0.1 ATP alone; Hypoxia 4.9 ± 1.5 ATP + EPO *vs* 5.4 ± 0.9 ATP alone) (Fig. 3.10).

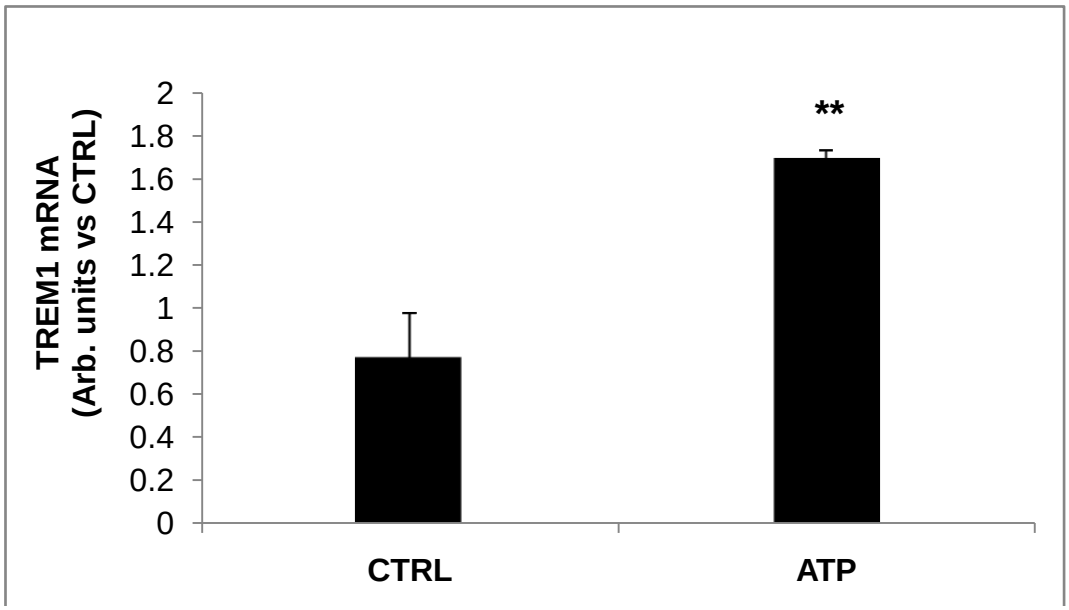


Figure 3.9: ATP induces TREM-1. MM6 cells were plated at a concentration of 25 X 10^4 cells/ml in 24 well plates and then treated with ATP 4mM for 3 hours. Cells were collected using TRIzol reagent. After RNA extraction and reverse transcription cDNA was analysed by Sybr Green qPCR using β–actin as housekeeping gene. Results are the mean ± SD of three samples analysed in duplicate. **P < 0.01 *vs* CTRL by Student's *t* test.

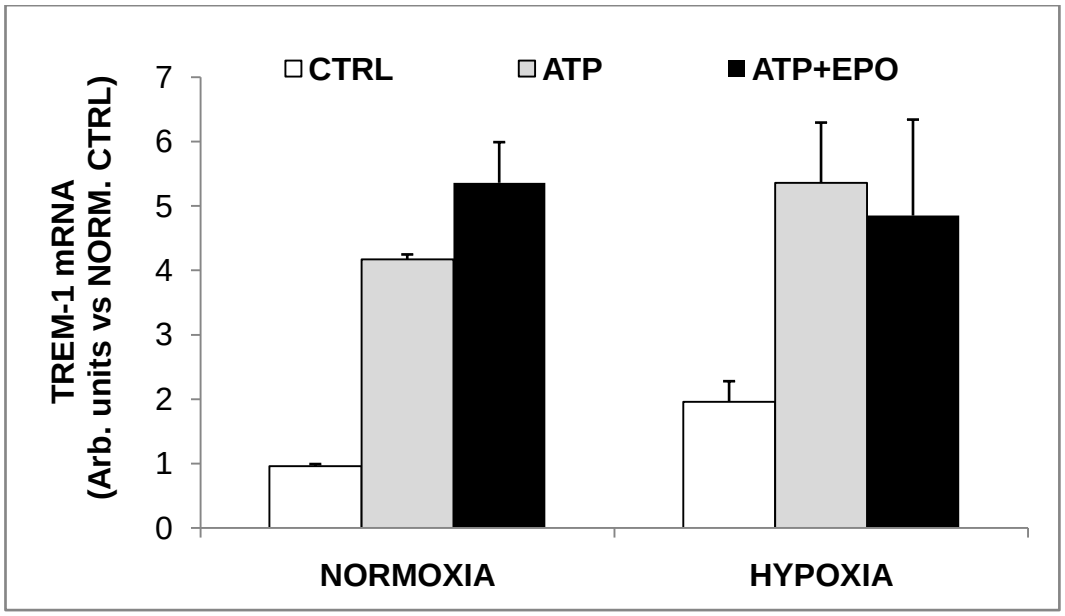


Figure 3.10: EPO does not inhibit TREM-1 induction. MM6 cells were plated at a concentration of 25 X 10^4 cell/ml in 24 well plates and treated with EPO 80 ng/ml. Half an hour later, cells were treated with ATP 4 mM for 3 hours. Cells were collected using TRIzol reagent. After RNA extraction and reverse transcription cDNA was analysed by Taqman qPCR using GAPDH as housekeeping gene. Results are the mean ± SD of three samples analysed in duplicate, and are expressed as arbitrary units *vs* a normoxic control. No significance observed by Student's *t* test between ATP and ATP+EPO.

In conclusion, ATP treatment induced TREM-1 expression but EPO did not show any inhibitory effect on TREM-1 *in vitro*, not confirming results obtained *in vivo* in the model of stroke.

3.4 Anti-inflammatory effect of EPO in human primary macrophages cells

The modulation of cytokines by EPO was also studied in primary human macrophage cells using different stimuli to induce cytokines through different pathways.

Peripheral blood was collected and PBMC were isolated. By density gradient centrifugation on isosmotic Percoll monocytes were collected and plated in 10 cm dishes at the concentration of 15 X 10^6 cells/dish. By addition of macrophage colony-stimulating factor (M-CSF), monocyte cells were differentiated to macrophages. Macrophages were then harvested and plated at a density of 2 X 10^5 cells/well in 96 well plates and left to adhere for 6 hours. Macrophages were treated with EPO or medium (control group). Two hours later they were further treated with different stimuli to induce cytokines expression. Cells were treated with IL1α 20 ng/ml (IL-1R), IL1β 20 ng/ml (IL-1R), R848 1 μg/ml (TLR7/8), LPS 1 ng/ml (TLR4) and PAM3 100 ng/ml (TLR1/2). Because R848 was dissolved in DMSO, this was used as negative control. No induction of TNFα was observed by DMSO showing that the effect of TNFα induction by R848 was not dependent on the DMSO vehicle. EPO did not decrease the expression of TNFα induced by different stimuli acting through different TLRs (Fig. 3.11).

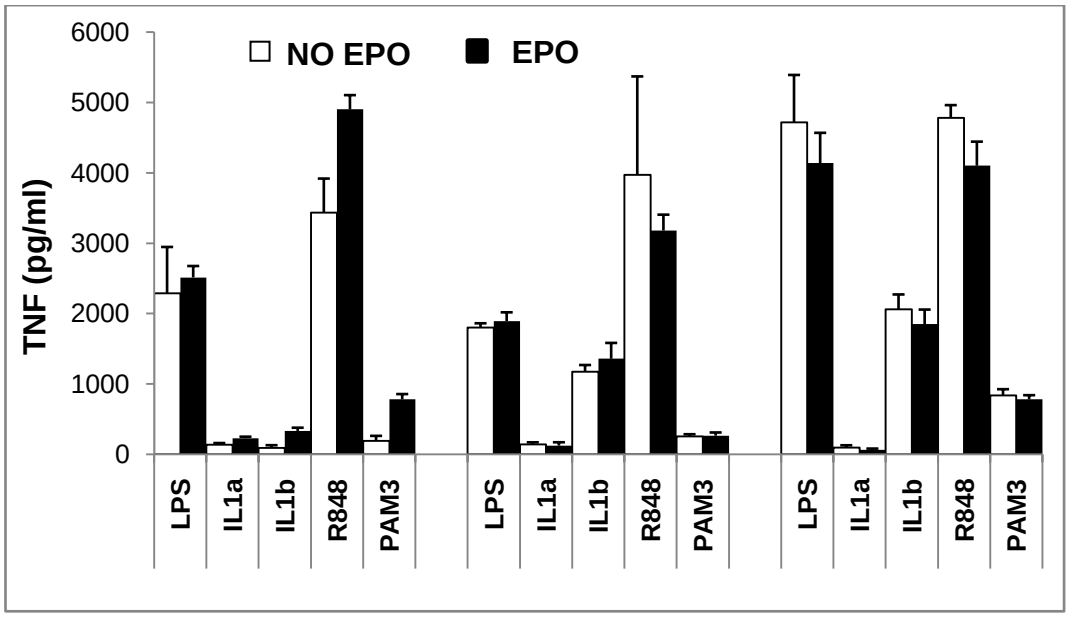


Figure 3.11: TNFα modulation by EPO following stimulation with different agents. Macrophages were plated in 96 well plates at a concentration of 2 X 10^5 cells/well. Macrophages derived from primary monocytes differentiated with M-CSF (100 ng/ml). After 6 hours, to allow cells to become adherent, medium was discarded and 2 hours before stimulation cells were treated with EPO. Stimuli used were: IL1α (20 ng/ml), IL1β (20 ng/ml), R848 (0.5 μg/ml), LPS (1 ng/ml), PAM3 (100 ng/ml). Results are the mean ± SD of three samples for each donor. Three donors were used for the experiment.

3.5 Role of stem/endothelial precursor cells from the bone marrow in EPO anti-inflammatory activity.

EPO mobilizes stem/endothelial progenitor cells from the bone marrow (Bahlmann et al., 2004). The hypothesis was that it is not possible to see a direct anti-inflammatory effect of EPO *in vitro* because the one observed *in vivo* is mediated by the recruitment into the circulation of a population of anti-inflammatory cells from the bone marrow.

In order to investigate this hypothesis, 5 CD1 male mice were treated with EPO (50 μg/Kg, i.p., daily, for three days), as reported (Heeschen et al., 2003), and 5 mice were treated with saline solution as control group. Previous experiments, carried out in the past in our laboratory, had confirmed that with this protocol it is possible to induce mobilization of $CD34^+$ stem cells into the circulation (Fig.3.12). At the end of the three day treatment, animals were sacrificed and circulating blood was collected with heparin. The blood was treated with different stimuli: LPS 10 ng/ml, LPS 1 μg/ml or Staphylococcus, in order to induce inflammatory cytokines. Samples were placed in the incubator 5% CO_2 for 24 hours. At the end of the incubation period, samples were centrifuged at 1200 rpm for 20 minutes and supernatants were collected for TNFα detection by ELISA (Scheme of the experiment showed in Fig. 3.13). TNFα was undetectable in all samples.

Therefore, a preliminary experiment was carried out to find the optimal dose of LPS and stimulation time. The experiment was repeated as described above, but just with 2 mice and without control. Triplicate blood samples were treated with two concentrations of LPS (1 and 10 μg/ml) and at two different time points of 4 hours and 24 hours. TNFα was measured by ELISA in supernatants. Since the lowest dose of 1 μg/ml at 4 hours induced TNFα at a good level, this was chosen for further experiments (Fig. 3.14 A). Finally, the experiment was repeated with 10 mice, 5 control and 5 treated with EPO, with LPS 1 μg/ml stimulation for 4 hours.

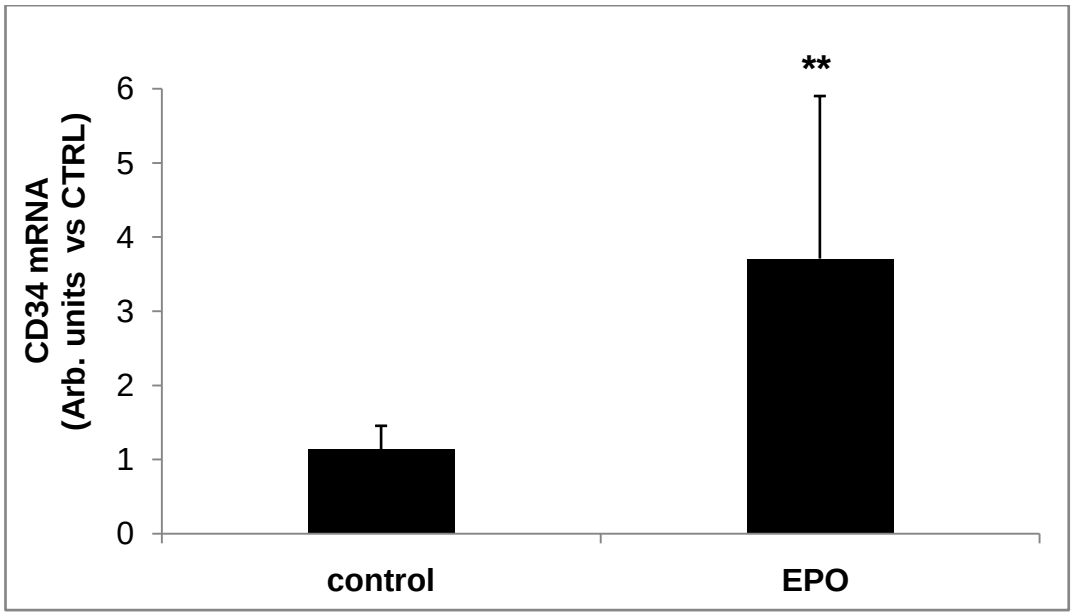


Figure 3.12: EPO mobilizes CD34+ cells in mice after 3 days of EPO treatment (previous unpublished experiment in the lab). CD1 mice (N = 11 per group) were injected subcutaneously with saline or rHuEPO (50 µg/Kg) for 3 consecutive days (Heeschen et al., 2003). Three hours after the last injection, peripheral blood was collected by cardiac puncture, mononuclear cells were separated by gradient centrifugation and total RNA was extracted. CD34 mRNA expression was measured by qPCR. HPRT1 was used as a housekeeping gene. Results were expressed as arbitrary units *vs* one control sample, and are the mean ± SD of 11 samples. **P< 0.01 by Student's *t* test.

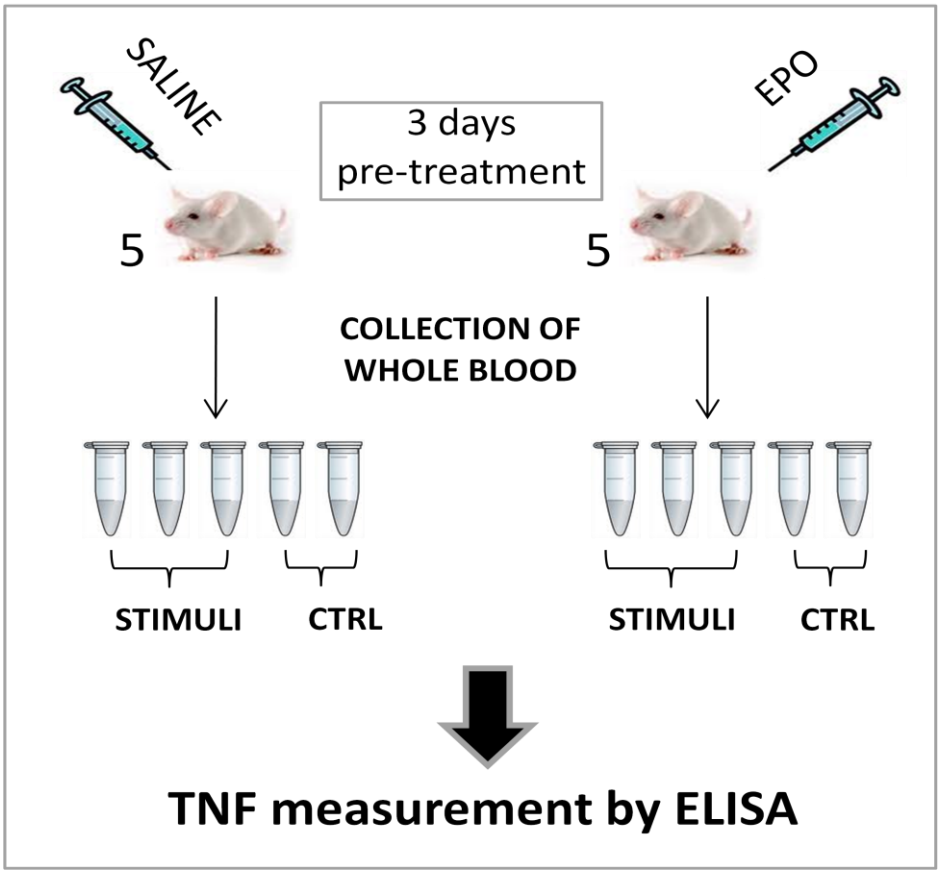


Figure 3.13: Overview of precursors mobilization by EPO *in vivo* in peripheral blood and cytokine induction *ex-vivo*. 5 mice CD1 were pre-treated daily with EPO and 5 mice with saline solution, as control group. After 3 days, mice were sacrificed and the peripheral blood was collected in heparinised tubes. The blood was treated with LPS in order to induce cytokines production. TNFα was measured in supernatants by ELISA after 24 hours.

Unfortunately, as in all other cases presented, EPO did not decrease the induction of TNFα and seemed to have a slight effect of induction of TNFα instead (Fig. 3.14 B).

In conclusion, this *ex-vivo* model to study the anti-inflammatory effect of EPO did not bring any positive results, implying that the hypothesis of precursor cells involved in mediation of the anti-inflammatory effect of EPO was not right. This was a further confirmation that EPO did not show any direct anti-inflammatory activity in vitro. On the other hand, EPO activated macrophages and slightly upregulated the induction of TNF in some specific conditions.

A

TNFα (pg/ml)		
	Mouse 1	Mouse 2
CTRL	< 10	< 10
1 μg 4 h	105.7	93.2
10 μg 4 h	163.3	120.1
1 μg 24 h	57.6	61.5
10 μg 24 h	169.1	92.2

B

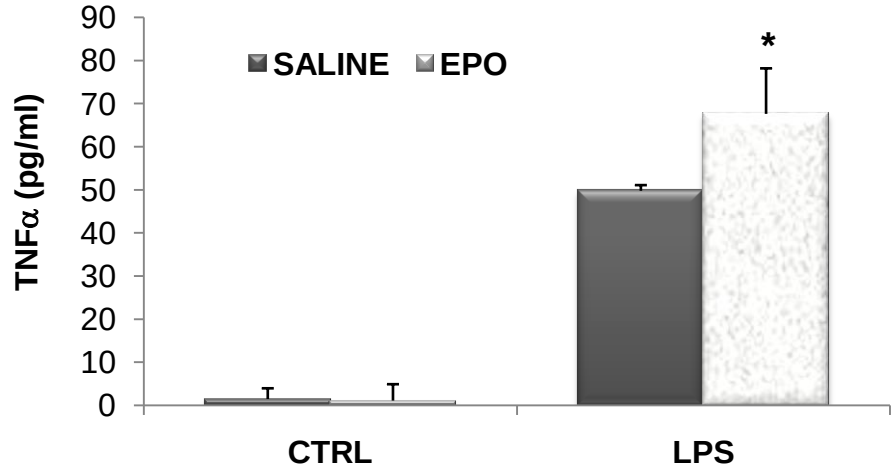


Figure 3.14: EPO mobilization of precursor cells does not modulate TNFα induced ex-vivo.

A. Preliminary experiment with 2 mice treated daily with EPO 50 μg/kg. After 3 days mice were sacrificed and the whole blood was collected and stimulated with LPS at the dose of 1μg/ml or 10 μg/ml for 4 or 24 hours. Supernatants were analysed by ELISA for TNFα.

B. 5 CD1 mice were treated with EPO 50 μg/kg and 5 with saline solution for 3 days. The peripheral blood was collected and treated with LPS 1 μg/ml for 4 hours. TNFα was detected by ELISA. There was no modulation of TNFα in mice pre-treated with EPO. Results are the mean ± SD of three samples analysed in duplicate. *P<0.05 by Student's *t* test *vs* CTRL.

3.6 Summary of chapter 3

The aim of this part of the work was to investigate whether EPO could have a direct anti-inflammatory effect *in vitro*.
So far, an anti-inflammatory effect of EPO was clearly observed in *in vivo* models of diseases by decreasing inflammatory cytokines. However, the anti-inflammatory effect of EPO *in vitro* is controversial. To date, the main hypothesis was that EPO might indirectly act as anti-inflammatory through inhibition of apoptosis.
In this work, several *in vitro* models of inflammation were used, trying to reproduce inflammatory pathways activated during diseases. Inflammatory cytokines, IL6 or TNFα, were studied.
The lack of EPO effect on cytokines induced by LPS was already reported. We confirmed that EPO did not have any effect on LPS-induced IL6, using PBMCs. Therefore, the effect of EPO was studied in other models of inflammation induced by HMGB1, NCL and ATP+LPS, the last one inducing the inflammasome. EPO treatment was unable to reduce cytokines induction in all the models investigated.
In addition, TREM-1, an inflammatory receptor triggering inflammation, was also investigated. In an *in vivo* model in which EPO was protective, TREM-1 was down regulated by EPO. Therefore, the hypothesis was that EPO could act with an anti-inflammatory effect by inhibition of this inflammatory receptor. TREM-1 was induced by ATP treatment. However, also in this case, EPO did not have any effect on TREM-1 inhibition.
Finally, different inductors of inflammation (IL1α, IL1 β, R848, LPS and PAM3) were used to stimulate primary macrophages. These stimuli induced TNFα but EPO did not decrease TNF expression. All these results supported the hypothesis that a direct anti-inflammatory effect of EPO could not be observed *in vitro.*
The last hypothesis investigated whether the anti-inflammatory effect of EPO could derive from the mobilization of endothelial precursor cells. As reported by Heeschen et al, this mobilization is obtained *in vivo* by treating mice for 3 days with EPO, but the role of these precursor cells is still

unclear (Heeschen et al., 2003). The whole blood of these mice was stimulated with LPS and an induction of TNF was observed.
However, once again, no anti-inflammatory effect of EPO was detected.

Chapter 4: EPO study on oligodendrocyte precursor cells differentiation

The expression of EPO has also been found in the brain in oligodendrocyte cells and EPOR was detected in oligodendrocytes at the same level as in neural cells (Sugawa et al., 2002). A previous *in vitro* study showed that exogenous EPO increased the number of myelin basic protein (MBP)-positive cells in oligodendrocytes (Sugawa et al., 2002). *In vivo*, in diseases like multiple sclerosis (MS), EPO is protective by decreasing inflammatory cytokines and improving the clinical score. However, it is unknown if EPO acts only with an anti-inflammatory mechanism or it also has a repair effect.

The goal of this chapter was to deeper investigate the effect of EPO on oligodendrocytes, by looking at myelin gene induction that can be related to a role of EPO on myelination.

To this aim, a line of OPCs from rats was used (provided by the collaboration with the Chernajovsky laboratory at Queen Mary University of London). These cells are able to differentiate to oligodendrocytes by withdrawal of growth factors and are considered a good tool to study myelination *in vitro* (Annenkov et al., 2011; Wang et al., 2011; Menon et al., 2005; Nguyen et al., 2003). A comparison study between primary culture and CG4 cells concluded that, even with some different characteristics, the two cells are mostly similar (Stariha and Kim, 2001). In addition, the same cell line modified to overexpress EPOR was also provided by the Chernajovsky lab, with the chance to study the mechanism of action of EPO in these cells.

Maturation of OPCs to oligodendrocytes can be observed by analysis of two of the main components of myelin: MBP and myelin oligodendrocyte glycoprotein (MOG), both of which are good markers of differentiation since are produced only by mature oligodendrocytes.

Scheme for experiments conducted in this chapter

In this chapter WT and genetically modified CG4 cells were used. CG4 cells are a line of rat oligodendrocyte precursors able to differentiate to oligodendrocytes. These cells were kept in an undifferentiated stage with the growth medium (GM) containing growth factors and the conditioned medium from neuronal B104 cells. When the differentiation medium (DM) was used instead, without growth factors, they differentiated to oligodendrocytes. The differentiation is morphologically characterized by the acquisition of several branched membrane processes and by a more rounded nucleus. In addition, as proof of differentiation to oligodendrocytes, some myelin genes like MOG and MBP can also be detected, since they are produced by mature oligodendrocytes only. Cells were plated at different concentrations as specified in each paragraph in poly-L-ornithine pre-coated plates. Cells were left in growth medium (GM), obtained as described in the method session, until they become sub-confluent, for about 1 or 2 days. Then, the medium was removed and cells washed twice with DMEM (without growth factors). At this time, the differentiation medium (DM) was added and, when required by the experimental plan, EPO treatments were started half an hour after differentiation began. The DM was changed every other day and also EPO treatments were repeated at that time. At the end of the experiments, cells were collected with TRIzol reagent and the RNA was extracted. After reverse transcription the cDNA was analysed for the genes of interest by qPCR. HPRT1 was used as housekeeping gene.

4.1 Effect of EPO on myelin gene induction

4.1.1 Effect of EPO on myelin gene induction in CG4 WT cells

The first preliminary experiment aimed to study MBP gene expression and the modulatory effect of EPO on it in CG4 WT cells.
CG4 WT cells were plated at a concentration of 8 X 10^4 cells/well in 24 well plates. When sub-confluent they were differentiated. According to Louis, these cells needed 2 days to start to acquire a phenotype typical of oligodendrocytes (Louis et al., 1992). Cells were also treated with EPO 80 ng/ml during differentiation. Every other day fresh EPO treatments were carried out and the medium was changed.
The experiment was stopped at different time points after 0, 2 and 4 days of differentiation. It was not possible to have a later time point because the cell number was already very high after 4 days. The initial cell concentration was chosen to match the one used in previous studies in our lab with the same cells in an undifferentiated stage. In theory, these cells should stop growing after 2 days of differentiation. However, the CG4 cells continued to grow up to 4 days of differentiation, making the concentration too high for these experiments.
After RNA extraction and reverse transcription, the cDNA was analysed by qPCR for MBP gene expression, using Taqman assays. The expression of MBP in these cells doubled after two days of differentiation compared to day 0 and the expression rose again 4 folds at day 4 compared to day 0. Unfortunately, no induction by EPO was observed at any of the time points analysed, showing that these cells were unresponsive to EPO (Fig. 4.1).
At first, we asked whether EPO did not have any effect because the cell number was too high after 4 days of differentiation and MBP gene expression had already entered a plateau phase, in which it was impossible to see any further induction. Therefore, the experiment was repeated using a lower initial cell concentration, and at a longer time point of 5 days. A higher dose of EPO was used this time. CG4 cells were plated in 24 well plates at a concentration of 3 X 10^4 cells/well.

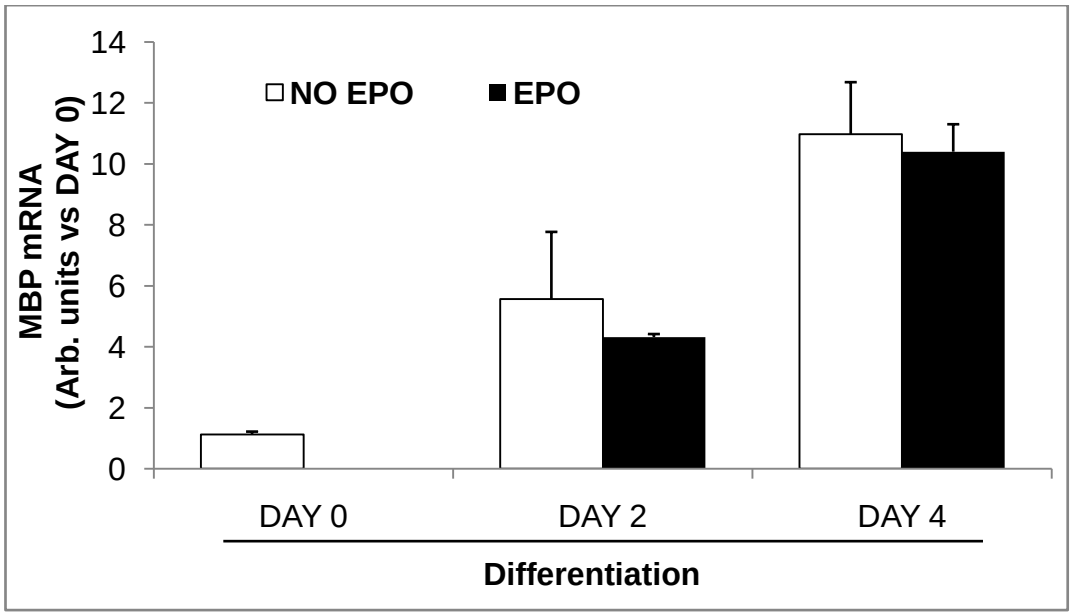


Figure 4.1: EPO does not induce MBP gene expression in CG4 WT cells during differentiation. CG4 WT cells were plated at a concentration of 8 X 10^4 cells/well in 24 well plates. The experiment was stopped at 0, 2 and 4 days from the beginning of differentiation. EPO treatment started at day 0 of differentiation and it was repeated every other day, at the same time of medium change. MBP gene expression was analysed by qPCR using HPRT1 as housekeeping gene. Results are the mean ± SD of three samples analysed in duplicate. MOG mRNA is expressed as arbitrary units *vs* one control at day 0. There was no effect of EPO on modulation of MBP gene expression at both time points considered.

The concentration was chosen after a few preliminary experiments trying to understand the behaviour of these cells during differentiation. After one day, cells were differentiated for 5 days and treated with EPO 400 ng/ml. MOG was increased after 5 days of differentiation showing that CG4 cells in culture had differentiated into oligodendrocytes. However, EPO treatment did not induce the expression of these genes in CG4 WT cells, even at the higher dose (Fig. 4.2).

Since in these experiments EPO did not have any effect in CG4 WT cells, we asked whether they expressed EPOR. EPOR is expressed in primary oligodendrocyte cells, as reported by Nagai (Nagai et al., 2001), and for that reason we had assumed that it was expressed by CG4 WT cells, although this had not been quantified. The cDNA obtained from the previous experiment was also analysed for EPOR gene expression by qPCR using Taqman assays. A very low expression of EPOR was found in CG4 WT cells (the fluorescence threshold cycle for EPOR amplification was higher than 35).

Therefore, we concluded that CG4 WT cells were not a good tool to study the effect of EPO on myelin gene expression because of the lack of EPOR.

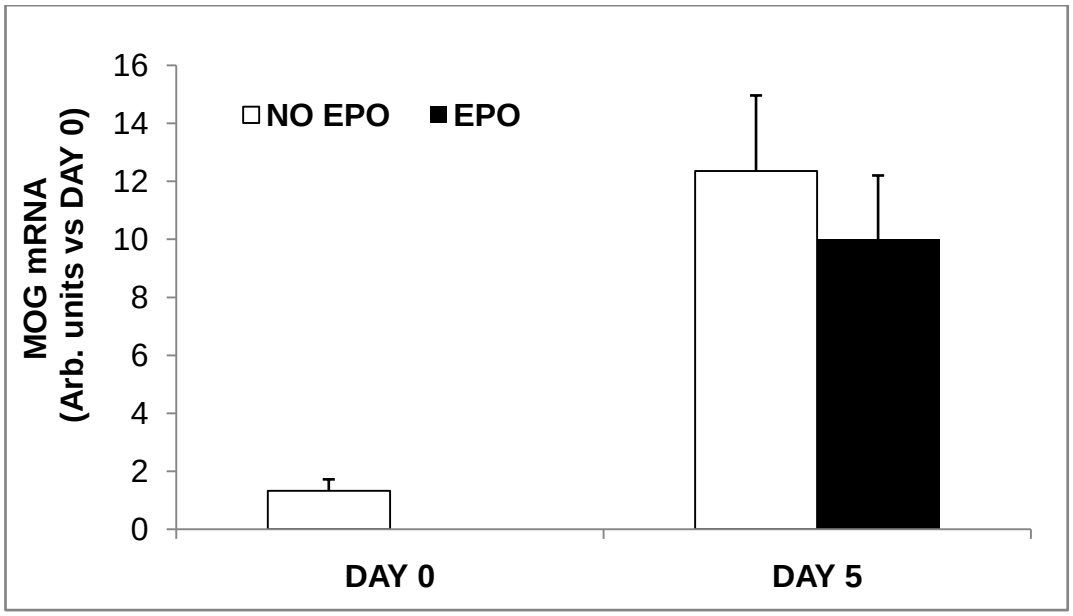


Figure 4.2: EPO does not induce MOG in CG4 WT cells at day 5 of differentiation. CG4 WT cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. Cells were treated with EPO 400 ng/ml, half an hour after the induction of differentiation. MOG gene expression was analysed by qPCR using HPRT1 as housekeeping gene. Results are the mean ± SD of three samples analysed in duplicate and expressed as arbitrary units vs NO EPO at day 0. No induction of MOG gene expression was found by EPO treatment after 5 days of differentiation.

4.1.2 Behaviour of CG4EPOR cells compared to CG4 WT cells

Through a collaboration with the Chernajovsky laboratory at Queen Mary in London, we obtained CG4 cells overexpressing EPOR (CG4EPOR). Since EPOR is expressed in primary oligodendrocytes (Sugawa et al., 2002) and induced in disease and during demyelination (Cho et al., 2012), CG4 cells modified to overexpress EPOR appeared to be a suitable model to study the effect of EPO.

However, before using CG4EPOR cells as a model of myelination, CG4EPOR and CG4 WT cells needed to be compared in terms of differentiation ability. An experiment was set up, at the time points of 0, 2 or 4 days of differentiation, to study the induction of myelin genes in both cell lines. Cells were plated at a density of 3×10^4 cells/well in 24 well plates. At the end of the experiment, MBP gene expression was analysed. CG4 WT and EPOR cells differentiated exactly in the same way, as seen by a similar MBP expression at all time points analysed (Fig. 4.3).

In conclusion, this experiment confirmed that CG4EPOR cells were a good tool to study EPO effect on myelin gene induction, since they behaved like CG4 WT cells in terms of differentiation ability.

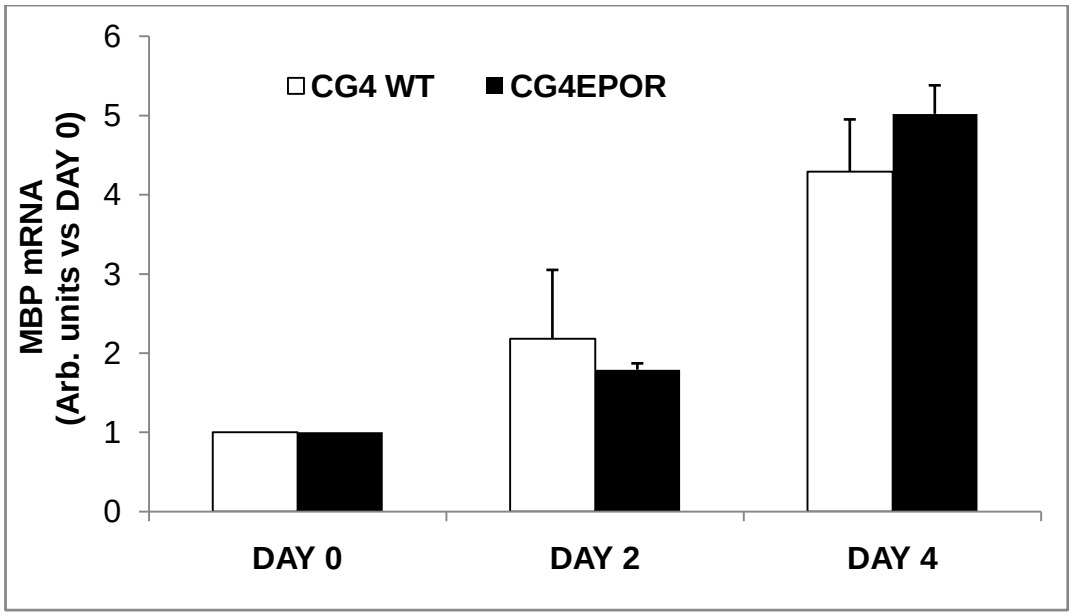


Figure 4.3: MBP gene expression during differentiation is the same in CG4 WT and CG4EPOR cells. CG4 WT and CG4EPOR cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. The experiment was stopped after 0, 1, 2 and 4 days from beginning of differentiation. MBP gene expression was analysed by qPCR using HPRT1 as housekeeping gene. Results are the mean ± SD of three samples analysed in duplicate. MBP mRNA is expressed as arbitrary units *vs* one control at day 0. No significant difference was observed by Student's *t* test between the two cell lines.

4.1.3 Effect of EPO on myelin gene induction in CG4EPOR cells

The first experiment was conducted to determine whether CG4EPOR cells were responsive to EPO, by studying myelin genes induction.
CG4EPOR cells were plated in 24 well plates at a concentration of 3 X 10^4 cells/well. The day after, cells were differentiated for 6 days. Half an hour after the addition of DM, cells were treated with EPO 80 ng/ml. MOG gene expression was analysed by qPCR. MOG mRNA was expressed as arbitrary units *vs* control at day 0 of differentiation. After 6 days of differentiation, EPO-induced MOG gene expression was 3.5 times greater than in differentiated cells without EPO treatment (Fig. 4.4). Better reagent preparation and improved laboratory techniques may explain the higher increase in MOG gene expression observed in this experiment compared to previous experiments.
In conclusion, CG4EPOR cells were responsive to EPO and EPO treatment increased MOG expression. Therefore, further studies were carried out to characterize better the effect of EPO.

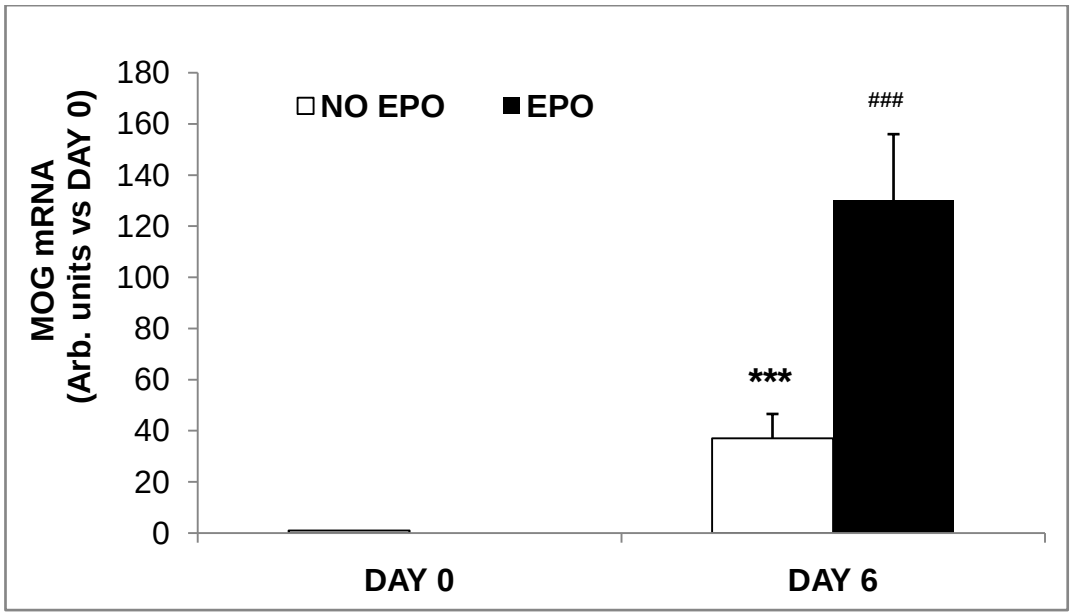


Figure 4.4: EPO increases MOG gene expression in CG4EPOR cells after 6 days of differentiation. CG4EPOR cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates pre-coated with poly-L-ornithine. EPO treatments, at the dose of 80 ng/ml, were done half an hour after adding the differentiation medium. MOG gene expression was evaluated by qPCR. MOG mRNA is expressed as arbitrary units vs one NO EPO sample at day 0. Results are the mean ± SD of three samples analysed in duplicate. ***P<0.001 *vs* day 0; $^{###}$P<0.001 *vs* day 6 NO EPO, by Student's *t* test.

4.2 Characterization of EPO effect on CG4EPOR cells

4.2.1 Effect of EPO on CG4 cells transduced with an empty vector

CG4EPOR cells, from Chernajovsky laboratory, were obtained from CG4 WT cells genetically modified by transduction with a lentiviral vector containing EPOR. A negative control was used to confirm that the effect of myelin gene induction by EPO we observed in these cells was specifically due to the expression of the receptor and was not a consequence of the lentiviral vector. The same CG4 cell line was modified by transfection with an empty lentiviral vector (CG4 EGFP). The previous experiment of induction of MOG and MBP gene expression by EPO was repeated in these cells.

CG4EGFP cells were plated at a concentration of 6 X 10^3 cells/well in 24 well plates (as explained in 4.2.2). Cells were differentiated for 5 days and treated with EPO 80 ng/ml. The cDNA was analysed by qPCR for MOG and MBP gene expression. There was no effect of EPO on both genes at day 5 but a good differentiation, shown by MOG and MBP gene induction, revealed that cells were definitely mature oligodendrocytes (MOG: 37.2 ± 2.7 NO EPO *vs* 40.6 ± 12.9 EPO) (Fig. 4.5 A); (MBP: 56.6 ± 12.7 NO EPO *vs* 47.5 ± 2.3 EPO) (Fig. 4.5 B).

This result showed that the induction of myelin gene expression by EPO in CG4EPOR cells was due to an overexpression of the receptor and not to the modification made with the lentiviral vector.

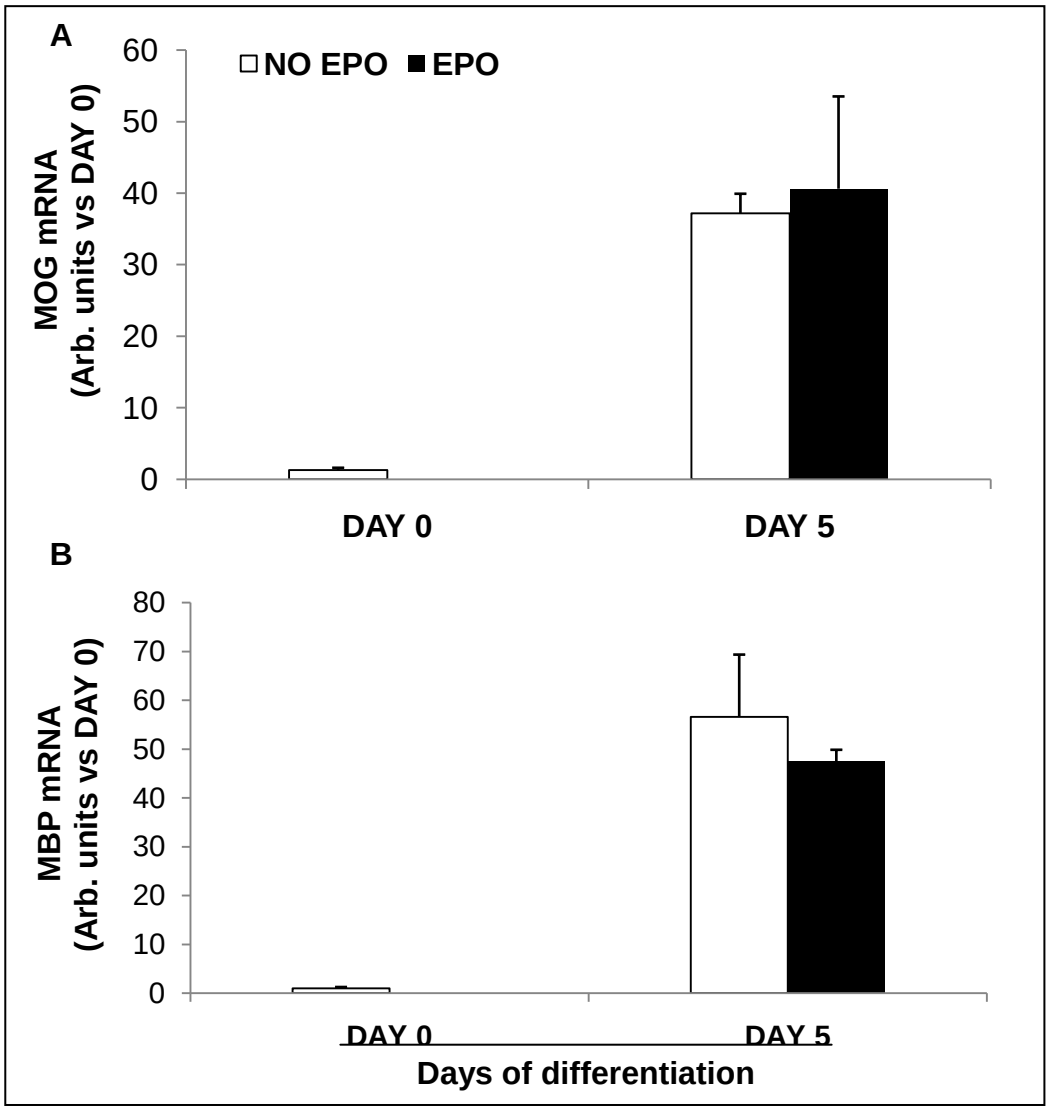


Figure 4.5: EPO does not have any effect on myelin gene induction in CG4 EGFP cells at day 5 of differentiation. CG4 EGFP cells were plated at a concentration of 6 X 10^3 cells/well in 24 well plates. Cells were differentiated for 5 days and treated with EPO 80 ng/ml. MOG (A) and MBP (B) gene expression were expressed as arbitrary units *vs* day 0. Results were the mean ± SD of three samples analysed in duplicate. EPO did not have any effect on myelin gene induction in CG4 EGFP cells, showing that EPOR is necessary to mediate this effect.

4.2.2 MOG and MBP dose-response in CG4EPOR cells

Before further investigating the effect of EPO on myelin gene induction in CG4EPOR cells, we did a dose-response experiment to find the optimal dose of EPO. CG4EPOR cells were plated in 24 well plates at a concentration of 6 X 10^3 cells/well. This concentration was chosen based on the work of Louis who suggested growing these cells in 10 cm petri dishes at a concentration of 1 X 10^5 cells/dish (Louis et al., 1992). Therefore, the cell concentration was strongly decreased to see if there was a large change in cell differentiation compared to the previous results. Cells were differentiated for 2 days, because after day 1 there were not enough to start the experiment. At the time of differentiation, cells were treated with EPO at several concentrations: 0.04 pg/ml, 4 pg/ml, 0.4 ng/ml, 8 ng/ml, 80 ng/ml and 400 ng/ml. The experiment was stopped at day 6 of differentiation. The cDNA obtained was analysed by qPCR for MOG and MBP gene expression. Significance was established by Dunnett's method comparing each dose of EPO with the control group (without EPO treatment).

At doses of EPO higher than 8 ng/ml, there was a plateau phase in the induction of both genes. All other doses, lower than 8 ng/ml, were not significantly different from the control. EPO increased MBP gene expression by a factor of about 3 (Fig. 4.6 A) and MOG by a factor of 5 (Fig. 4.6 B), compared with untreated differentiated cells at the same time point ($P<0.001$). The myelin gene induction for both high and low initial cell concentrations was similar. Since all concentrations of EPO higher than 8 ng/ml had the same effect of induction of myelin genes, the dose of 80 ng/ml was chosen for further experiments as it was the dose previously used *in vitro* to study EPO in neuroprotection (Mengozzi et al., 2012) (Cho et al., 2010).

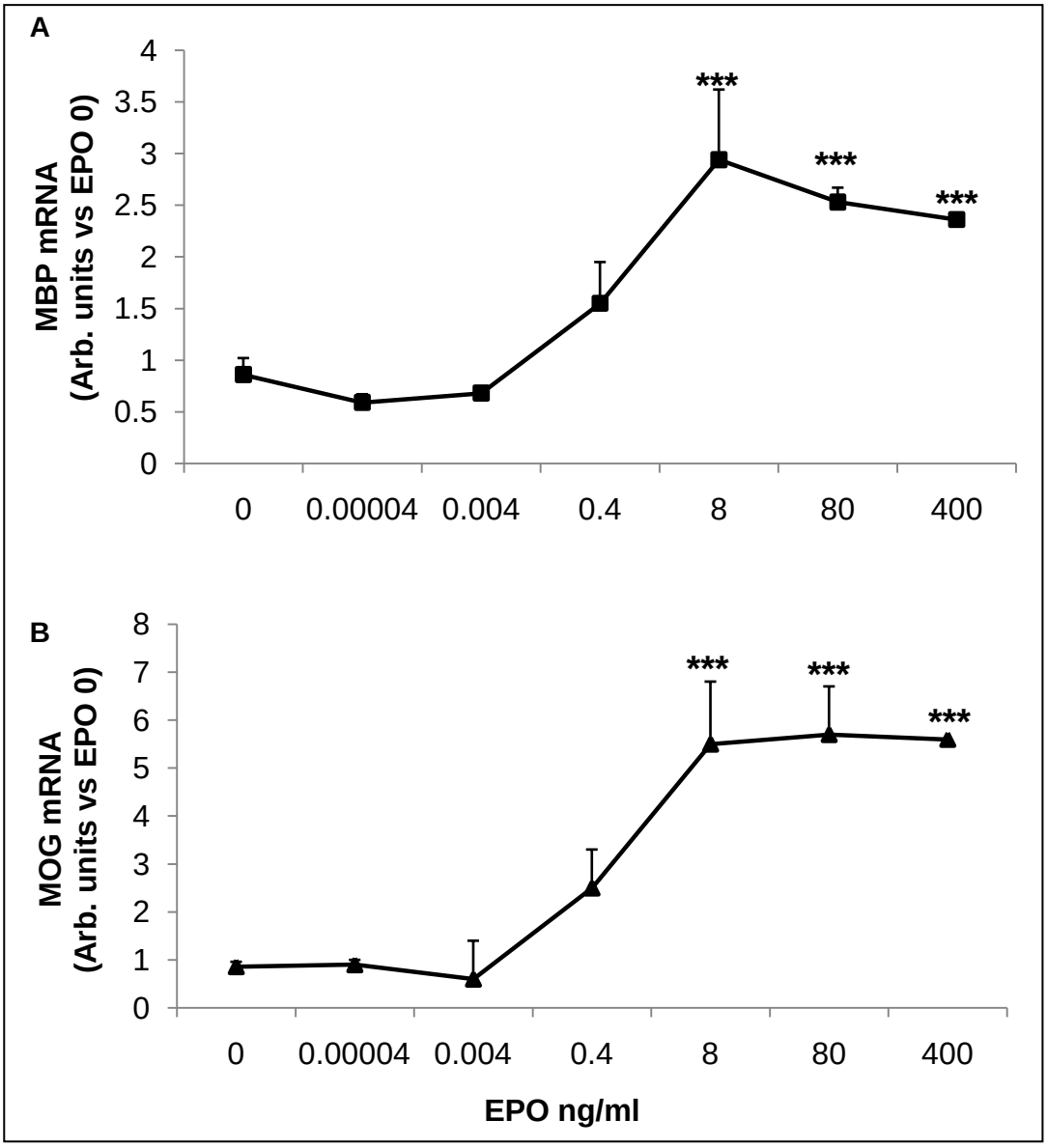


Figure 4.6: Dose-response of myelin gene expression in CG4EPOR cells treated with EPO. CG4EPOR cells were plated at a concentration of 6 X 10^3 cells/well in 24 well plates. Cells were differentiated and treated with EPO at the indicated concentrations for 6 days. MBP (A) and MOG (B) gene expression was analysed by qPCR. Results are the mean ± SD of three samples analysed in duplicate and expressed as arbitrary units *vs* one sample of the untreated group (EPO 0 ng/ml in the graph). ***P<0.001 by Dunnett's method *vs* untreated (EPO 0 ng/ml in the graph). At doses higher than 8 ng/ml EPO significantly induces myelin genes.

4.2.3 Time course of myelin gene expression in CG4EPOR cells treated with EPO

The dose of EPO 80 ng/ml was chosen to induce myelin genes in oligodendrocyte precursor cells. The next step was to study the time-course of this gene induction.

CG4EPOR cells were cultured in 24 well plates at an initial concentration of 3 X 10^4 cells/well. Cells were differentiated and treated with EPO 80 ng/ml. Different time-points were established at 0, 2, 4 and 6 days of differentiation. By qPCR the expression of MOG and MBP was analysed. Myelin gene induction by EPO started already at day 2 of differentiation when the expression of MBP was 2.3 times greater in the group treated with EPO than in the untreated group. MOG expression was induced by EPO of a factor of 4. EPO treatment increased MBP gene expression by a factor of about 4 at day 4 of differentiation. At day 6 of differentiation, this induction by EPO slightly decreased to about 2.7 folds more than untreated cells (Fig. 4.7 A). The analysis of MOG gave results really comparable to the previous ones. At day 4, MOG induction in EPO-treated cells was almost 4 times greater than the cells not treated with EPO. At day 6, the induction by EPO was greater by a factor of about 3.5 (Fig. 4.7 B).

In conclusion, EPO induced both MBP and MOG gene expression at day 4 and 6 of differentiation. This effect was slightly better at day 4 compared to day 6 of differentiation for both genes analysed.

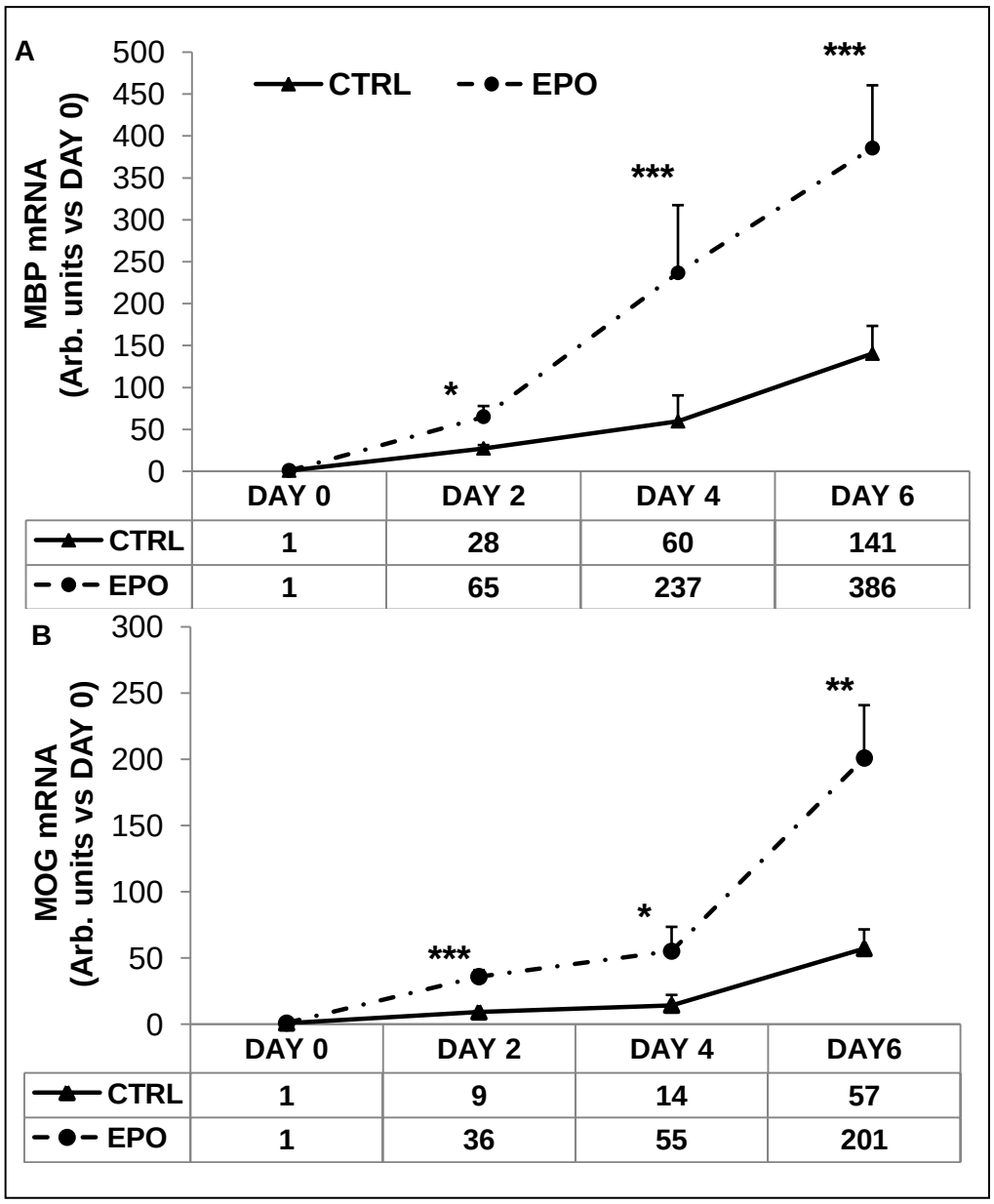


Figure 4.7: Time-course of MOG and MBP gene expression in CG4EPOR cells treated with EPO 80 ng/ml. CG4EPOR cells were plated at 6 X 10^3 cell/well in 24 well plates in GM. Cells were differentiated and treated with EPO 80 ng/ml during 6 days of differentiation. Experiments were stopped at the time points of 0, 2, 4 and 6 days of differentiation. MBP (A) and MOG (B) gene expression was analysed by qPCR. Results are the mean ± SD of 4-6 samples, from two different experiments, analysed in duplicate. *P<0.05, **P<0.01, ***P < 0.001 by Student's *t* test *vs* the respective no EPO group.

4.2.4 Protein expression

To further confirm that EPO is involved in oligodendrocyte differentiation, the induction of MOG protein by EPO was also investigated. CG4EPOR cells were plated in Petri dishes at the concentration of 10^6 cells/dish. Cells were differentiated with DM for 3, 6 and 9 days and they were treated with or without EPO 80 ng/ml during differentiation. CG4 cells were also plated at the same concentration and treated with or without EPO for 9 days of differentiation. At the established end points, supernatants were discarded and cells were lysed with lysis buffer, as described in the method session.

By western blot (WB), the expression of MOG protein in the different samples was analysed. Samples were run in a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins with different molecular weights. Afterwards, proteins from the gel were transferred into a nitrocellulose membrane. The membrane was first incubated with a mouse anti-MOG antibody, Z12 hybridoma, then with a goat anti-mouse antibody. MOG expression can be observed in the WB at the molecular weight of 28 kDa.

At 6 days of differentiation, it was already possible to observe a good induction of protein in CG4EPOR cells in the group treated with EPO compared to the untreated one, in which the protein was weakly expressed. This induction was also increased after 9 days of differentiation in the group treated with EPO compared to the untreated one. This induction was not confirmed in CG4 WT cells were EPO did not show any effect after 9 days of differentiation (Fig. 4.8). Validation was done using β-actin as control. This experiment was a further validation that EPOR is required by EPO to induce MOG gene expression and this induction is also translated in a greater protein expression.

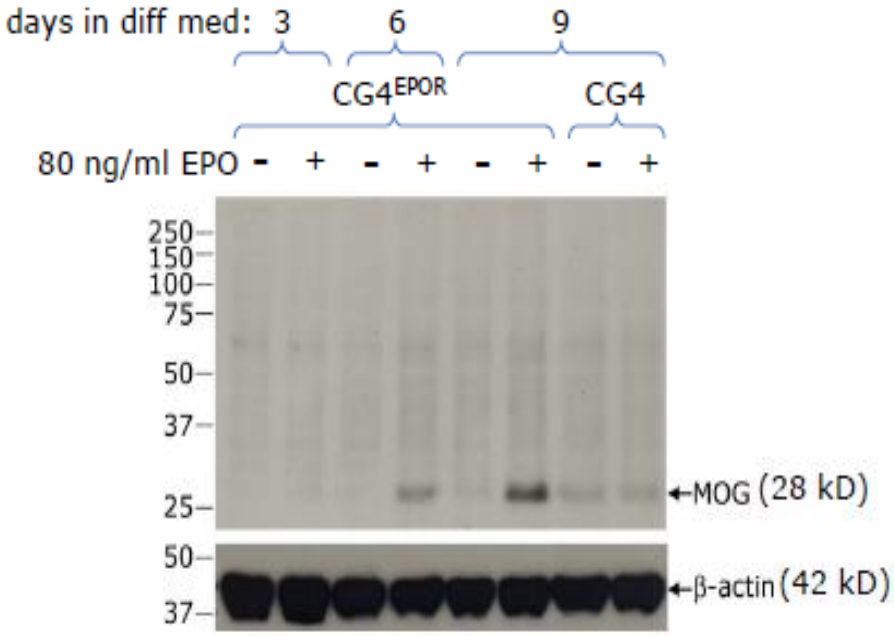


Figura 4.8: MOG protein expression is increased by EPO treatment after 6 and 9 days of differentiation. CG4EPOR cells were differentiated for 3, 6 and 9 days and treated with EPO 80 ng/ml during differentiation. CG4 cells were also differentiated for 9 days. Proteins were separated in a 10% SDS-PAGE gel and incubated with anti-MOG Z12 1:50 and goat-anti-mouse IgG-HRP 0.4 μg/ml. EPO increased MOG protein expression, only in cells overexpressing EPOR, as analysed by WB (MW 28). In CG4 cells EPO is not able to induce the expression of the protein. β-actin was used as control (MW 42 kD). This is a confirmation that EPO, through EPOR, not only induces myelin genes but also MOG protein expression.

4.2.5 Comparison between EPO and the myelinating agent leukemia inhibitory factor (LIF)

This experiment aimed to confirm the responsiveness of CG4EPOR cells to an agent known to be involved in the maturation of oligodendrocyte precursor cells. Leukemia inhibitory factor (LIF) is a pro-myelinating factor *in vitro* in oligodendrocyte cells (Ishibashi et al., 2006) and *in vivo* in the cuprizone model of demyelination (Marriott et al., 2008). According to Ishibashi (Ishibashi et al., 2006), myelination was increased by low concentration of LIF (< 5 ng/ml) but high concentrations (> 5 ng/ml) were inhibitory. If LIF induces differentiation in these cells (as shown by myelin gene and protein induction), it would be a further confirmation that CG4EPOR cells are a reliable *in vitro* model of myelination.

The effect of EPO (400 ng/ml) was compared with LIF 0.2 ng/ml, since this dose lies in a range known to be myelinating (Ishibashi et al., 2006). Cells were plated in 24 well plates at a concentration of 6 X 10^3 cells/well. At the differentiation time, cells were treated with EPO or LIF. The experiment was run for 6 days of differentiation. In this experiment the induction of MOG in differentiated cells, without any treatment, was about 27 folds greater than the control (1.4 ± 0.6 day 0 vs 27.8 ± 4.5 day 6; P<0.001) (Fig. 4.9). LIF induction of MOG was 3.6 times more than at day 6 without any treatment (101.1 ± 5 LIF *vs* 27.8 ± 4.5 day 6; P< 0.001) but EPO induction of MOG was about double that of LIF (211.1 ± 6.2 EPO *vs* 101.1 ± 5 LIF; P< 0.001) and therefore 7.6 times more compared to day 6 without any treatment (211.1 ± 6.2 EPO *vs* 27.8 ± 4.5 day 6; P< 0.001) (Fig. 4.9).

In conclusion, CG4EPOR cells are responsive to EPO as well as to LIF for myelin gene induction. Since LIF is a well known myelinating agent, this result shows that the observed EPO effect in these cells may be relevant and might translate into a myelinating effect.

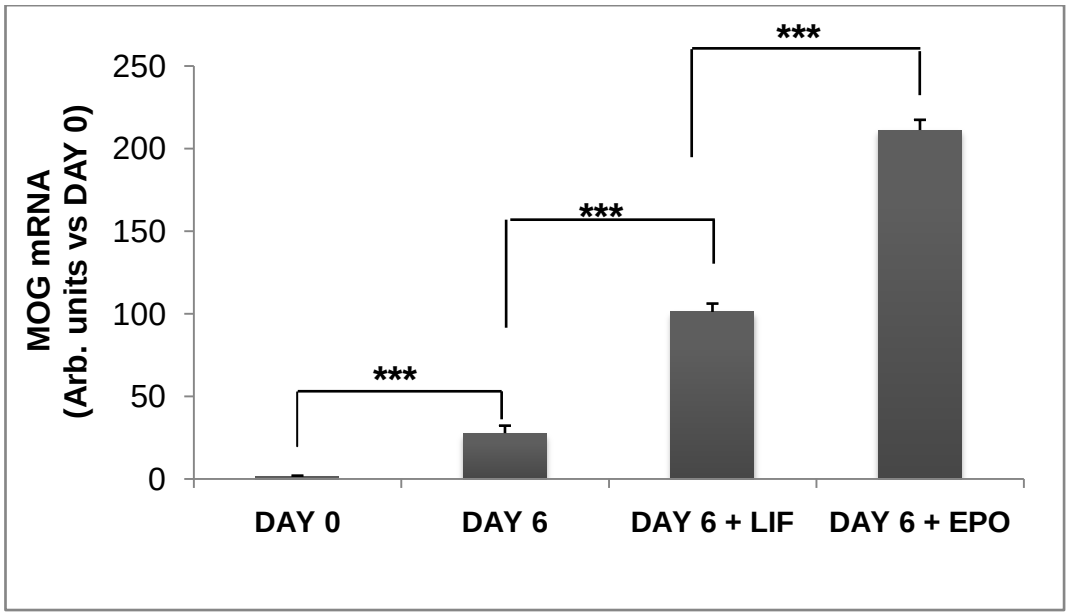


Figure 4.9: Both EPO and LIF induces MOG gene expression at day 6 of differentiation. CG4EPOR cells were plated at a concentration of 6 X 10^3 cells/well in 24 well plates. Cells were differentiated and treated with EPO 400 ng/ml or LIF 0.2 ng/ml for 6 days of differentiation. By qPCR, MOG gene expression was analysed. Results are the mean ± SD of three samples analysed in duplicate. ***P<0.001, by Student's *t* test. CG4EPOR cells are responsive to the induction of myelin gene expression by both EPO and LIF.

4.2.6 Induction of cell proliferation by EPO

Another question to be investigated was whether the increasing effect of EPO on myelin gene expression might be due to an increase of the cell number. To this aim, the proliferative effect of EPO in CG4EPOR cells was studied in both undifferentiated and differentiated cells, using the Cell Titer Blue (CTB) assay. This is a fluorescent method used to estimate viable cell number, and therefore can be used to evaluate cell proliferation.

CG4EPOR cells were plated in 96 well poly-L-ornithine pre-coated plates at different concentrations: 1,250, 2,500, 5,000, 10,000, 20,000 cells/well. After one day, cells were treated with EPO 80 ng/ml for two days. The experiment was stopped by removal of 100 μl of medium from each well and addition of 20 μl of CTB. After 4 hours, the fluorescence was measured using a fluorescence microplate reader (Synergy HT, Biotek) with excitation 530/25 nm and emission 590/40 nm filters.

The result showed no effect of EPO on induction of cell proliferation at all concentrations analysed (Fig. 4.10).

The experiment was also repeated with differentiated cells, since the effect of EPO on myelin genes was always observed during differentiation.

CG4EPOR cells were plated at a concentration of 8 X 10^3 cell/well in 96 well plates pre-coated with poly-L-ornithine. Cells were differentiated and treated with a high concentration of EPO (400 ng/ml) to be sure that even a high concentration did not have any effect on oligodendrocyte proliferation. The experiment was stopped at day 4 of differentiation. The result showed no effect of EPO on cell proliferation even in these differentiating cells (Fig. 4.11).

In conclusion, the observed effect of EPO on myelin gene induction was not a consequence of increased cell number due to induction of cell proliferation.

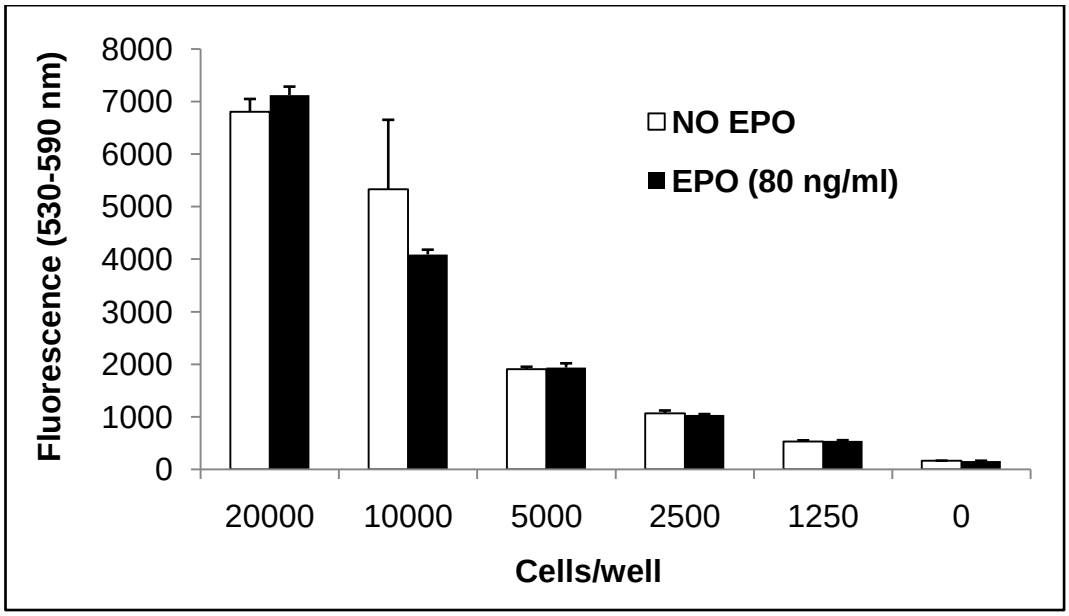


Figure 4.10: EPO does not increase the cell number in cultures of undifferentiated CG4EPOR cells. CG4EPOR cells were plated at different concentrations (20,000, 10,000, 5,000, 2,500 and 1,250 cells/well) in 96 well plates. Cells were kept in GM and treated with EPO 80 ng/ml for 2 days. After 2 days, 20 μl of CTB were added and the cell viability was assayed by measuring the fluorescence with a microplate reader with excitation 530/25 nm and emission 590/40 nm filters. Results are the mean ± SD of quadruplicate samples. EPO did not have any effect on cell proliferation.

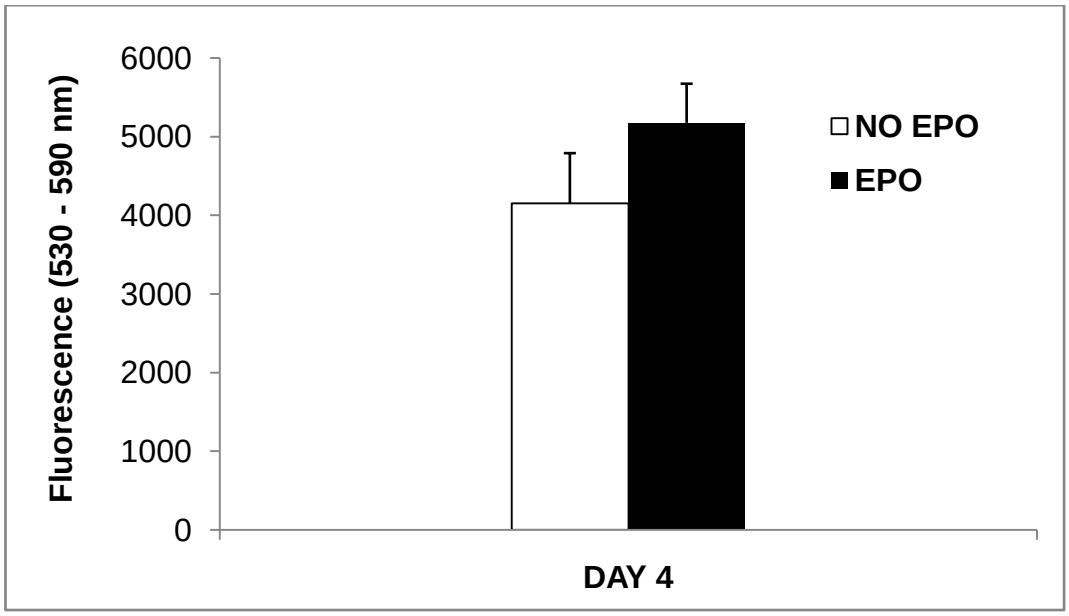


Figure 4.11: EPO does not increase the cell number in cultures of differentiating CG4EPOR cells. CG4EPOR cells were plated at a concentration of 8 X 10^3 cells/well in 96 well plates. The day after cells were differentiated using DM and treated with EPO 400 ng/ml. At day 2 the medium was changed and the treatment was repeated again. At day 4, 20 μl of CTB were added and after 4 hours the resulting fluorescence was measured using a fluorescence microplate reader with excitation 530/25 nm and emission 590/40 nm filters. Results are the mean ± SD of 4 samples for each group. No induction of cell proliferation was observed by EPO treatment.

4.3 Effect of EPO on induction of myelin genes in primary oligodendrocytes

By a collaboration with Mark Kotter laboratory in Cambridge, the effect of EPO on myelin gene induction in primary oligodendrocyte cells was also investigated. OPCs were isolated from rat pups, differentiated and then collected after 0, 2 and 4 days of differentiation. Samples were also treated during differentiation with EPO 80 ng/ml. MOG and MBP gene expression was evaluated by qPCR, using the beta-2 microglobulin as housekeeping gene.

Unfortunately, no induction of MOG and MBP by EPO was observed, at any time points (Fig. 4.12). A preliminary dose response experiment was also done to study the effect of EPO and check that the lack of EPO effect was not due to a too low concentration of EPO in the treatment. Several doses of EPO were used: 40 ng/ml, 80 ng/ml, 160 ng/ml, 320 ng/ml. EPO did not show any effect on induction of MOG or MBP at any dose used (Fig. 4.13).

The expression of EPO receptor in these cells was also analysed and compared with the expression of the receptor in CG4EPOR cells.

By qPCR, it was found that primary OPCs have an expression of EPOR that is about 250 folds less than the CG4EPOR cell line (Fold decrease 0.004 OPCs vs CG4EPOR). It is likely that the expression of EPOR in physiological conditions in primary OPCs is too low to have an effect of EPO in these cells.

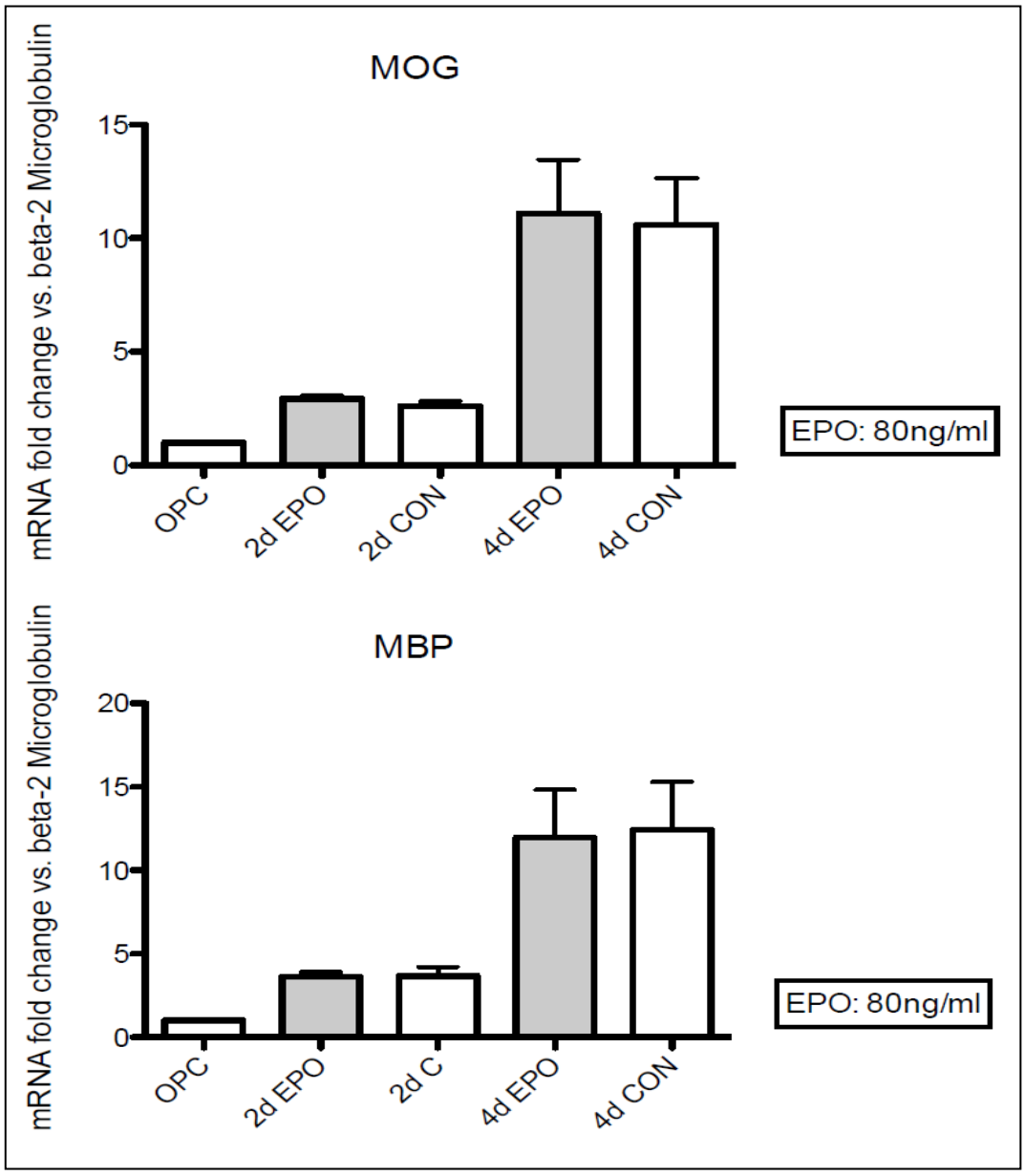


Figure 4.12: EPO does not have any effect on myelin genes induction in primary oligodendrocyte cells. Primary OPCs were treated for 2 and 4 days of differentiation with EPO 80 ng/ml or without EPO (CON). MOG and MBP gene expression were analysed by qPCR using beta-2 Microglobulin as housekeeping gene. No effect of EPO on both myelin gene induction was found.

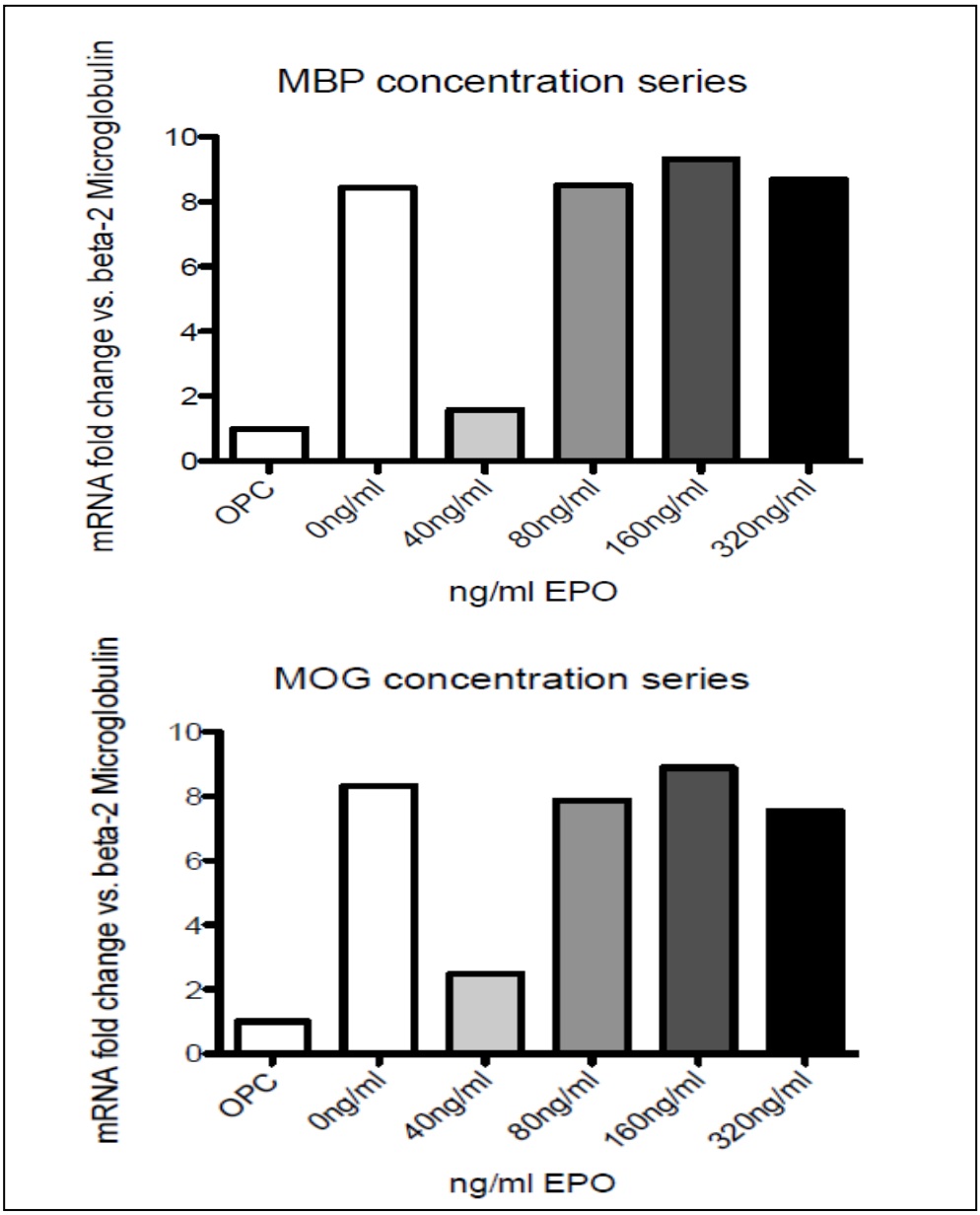


Figure 4.13: EPO does not have any effect on induction of MOG and MBP at any of the different doses used. A dose-response of EPO was done in primary OPCs at the time point of 4 days of differentiation. Cells were treated at the indicated doses. No effect of EPO was found.

4.4 Summary of chapter 4

The goal of this chapter was the investigation of the effect of EPO on induction of myelin genes. CG4 cells, a line of oligodendrocyte precursor cells, were used.

EPO did not have any effect on the induction of MOG and MBP gene expression in the CG4 WT cell line that did not express EPOR. However, EPO induced MOG and MBP gene expression in CG4EPOR cells of about 2.5 - 4 folds compared to untreated cells at 2, 4 and 6 days of differentiation, as analysed by qPCR. CG4EPOR cells were obtained from CG4 WT cells by transfection with a lentiviral vector to overexpress EPOR.

Although the effect of EPO was at a plateau phase starting from the dose of 8 ng/ml, the dose chosen in these experiments was 80 ng/ml since this is the one usually used *in vitro* to study EPO in neuroprotection.

By western blot also an induction of MOG protein expression was found after 6 days of differentiation and even increased after 9 days.

No effect of EPO was found in CG4 cells modified with an empty vector showing that the effect of myelin gene induction was not a consequence of the transfection.

The effect of EPO was not a consequence of an increase in the number of oligodendrocyte cells, as demonstrated by the cell viability assay.

In addition, CG4EPOR cells were responsive to another myelinating agent, LIF. Since LIF induced MOG gene expression in CG4EPOR cells, this would support the hypothesis that the effect of EPO on induction of myelin genes could be translated into a myelinating effect.

Finally, EPO effect was studied in primary oligodendrocytes to confirm the results so far obtained in the cell line. Unluckily, no effect of EPO on induction of myelin genes was found in primary cells, as studied at 2 and 4 days of differentiation and with different EPO concentrations. Maybe the lack of EPO effect in these cells was due to a low expression of EPOR in primary cells in physiological conditions.

Chapter 5: Erythropoietin receptor (EPOR) involvement in mediation of myelin gene induction by EPO

The receptor mediating EPO effect in neuroprotection is still an open issue. As discussed in the introduction, it is controversial whether the effect of EPO is mediated by the homodimeric EPOR or another unknown receptor. In fact derivatives of EPO that do not bind EPOR are still neuroprotective. In addition, the issue that EPO is active on non hematopoietic cells has been questioned and there is even a debate on whether EPOR is functional in non hematopoietic cells (Sinclair et al., 2010) (Ghezzi et al., 2010).
The aim of this chapter was therefore to demonstrate that the induction of myelin genes by EPO correlated with the expression of EPOR. To this purpose, induction of myelin genes by EPO was studied:

i) in CG4 WT cells, in which we tried to induce higher levels of EPOR;
ii) in a clone of CG4 WT cells, obtained by limiting dilution, in which the expression of EPOR was higher than in the cell line.
iii) in clones of CG4EPOR expressing different levels of EPOR, obtained by limiting dilution.

5.1 Induction of EPOR in CG4 WT cells

It is possible to induce EPOR expression *in vitro* by several inductors like EPO (Beleslin-Cokic et al., 2011), hypoxia (Beleslin-Cokic et al., 2011) and NO (Chen et al., 2010).

In the first experiment we tried to induce EPOR by EPO in normoxic or hypoxic conditions. CG4 WT cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. The day after cells were treated with or without EPO 80 ng/ml in normoxic or hypoxic conditions (5% O_2). The experiment was stopped after 48 hours, and then cells were collected, as reported in the method section. EPOR expression was measured by qPCR with the Taqman assays. Surprisingly, no expression of EPOR was detected neither in normoxic nor in hypoxic conditions.

Subsequently, the experiment was repeated keeping cells in a lower oxygen concentration and with NO stimulation, since it has been shown that NO in hypoxia induced EPOR production in neuronal cells (Chen et al., 2010). Cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates and the following day were treated with EPO 80 ng/ml, NO 30 μM or EPO plus NO (half an hour later). This scheme of treatment was repeated in normoxic or hypoxic (1% O_2) conditions. The experiment was stopped after 24 hours, cells were collected and samples were analysed by qPCR for EPOR. This time there was an induction of EPOR but only dependent on hypoxia stimulation (arbitrary units: 4.8 ± 1 hypoxic control *vs* 0.8 ± 0.2 normoxic control) (Fig. 5.1). In the hypoxic group treated with EPO there was a decrease of EPOR expression compared to the hypoxia alone (4.8 ± 1 hypoxic control *vs* 2.6 ± 0.4 hypoxia plus EPO; $P< 0.05$). NO did not show any effect, neither associated with hypoxia alone nor with EPO (Fig. 5.1).

In conclusion, the only induction of EPOR obtained in these experiments was with hypoxia 1% O_2. This scheme was used for further investigations.

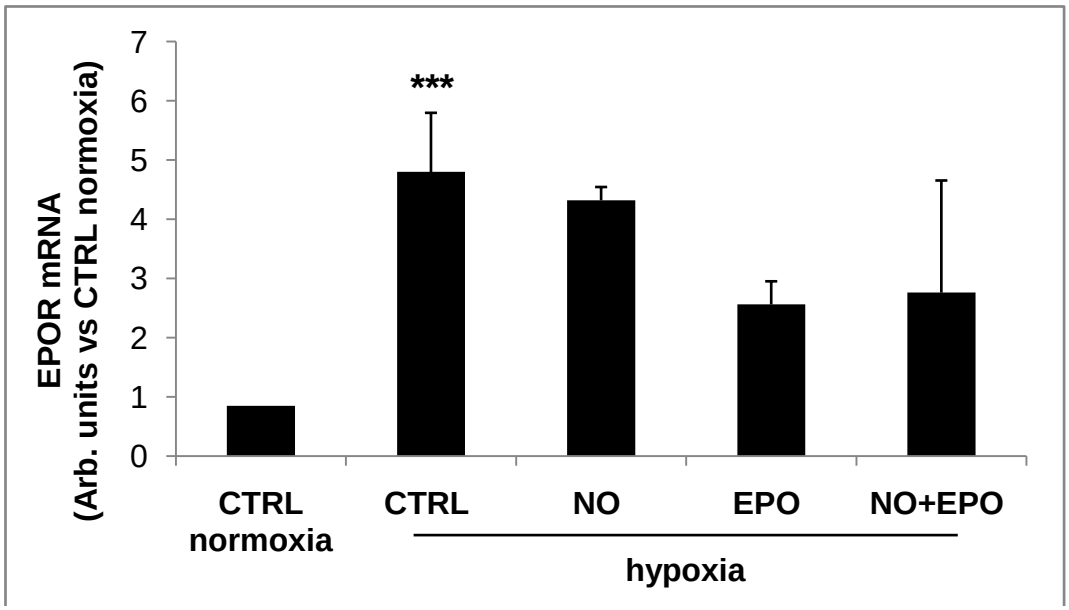


Figure 5.1: Hypoxia slightly induced EPOR expression in CG4 WT cells. Cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. The following day, cells were treated with EPO 80 ng/ml, NO 30 μM or EPO plus NO (30 min after EPO treatment). Cells were kept in normoxic or hypoxic conditions with 1% O_2. The experiment was stopped after 24 hours. Three samples for each experimental condition were analysed in duplicate by qPCR for EPOR expression. Results were expressed as arbitrary units ± SD *vs* control in normoxia. ***P< 0.001 by Student's *t* test. Hypoxia 1% O_2 slightly induces EPOR gene expression.

5.2 EPO effect on MOG gene expression in CG4 WT cells with hypoxia-induced EPOR

As shown in the previous experiment, hypoxia 1% O_2 was able to induce EPOR expression of about 5 folds in 24 hours. Hypoxia is not the best condition to study the induction of myelin genes since it can damage oligodendrocytes and white matter as observed in stroke (Pantoni et al., 1996). However, an experiment was set up to study EPO effect on MOG gene induction in CG4 WT cells after induction of EPOR. Cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. The following day, they were differentiated under hypoxic conditions (1% O_2) and treated with EPO. These cells were compared to those prepared under different conditions: undifferentiated, differentiated in normoxic and hypoxic conditions. In all cases, differentiation was stopped after 5 days. The cDNA was analysed by qPCR for MOG gene expression. As expected, there was a lower MOG induction in samples differentiated in hypoxia compared to the samples in normoxic conditions (65.2 ± 20.4 differentiated in hypoxia *vs* 119.3 ± 19 differentiated in normoxia; $P<0.05$) (Fig. 5.2). Moreover, in the group treated with EPO there was no difference in MOG induction compared with the hypoxia alone.

In conclusion, even when EPOR expression was increased by hypoxia, EPO had no effect on MOG gene expression induction in CG4 WT cells. This suggests that the induction of EPOR was too low to see an effect of EPO in this model.

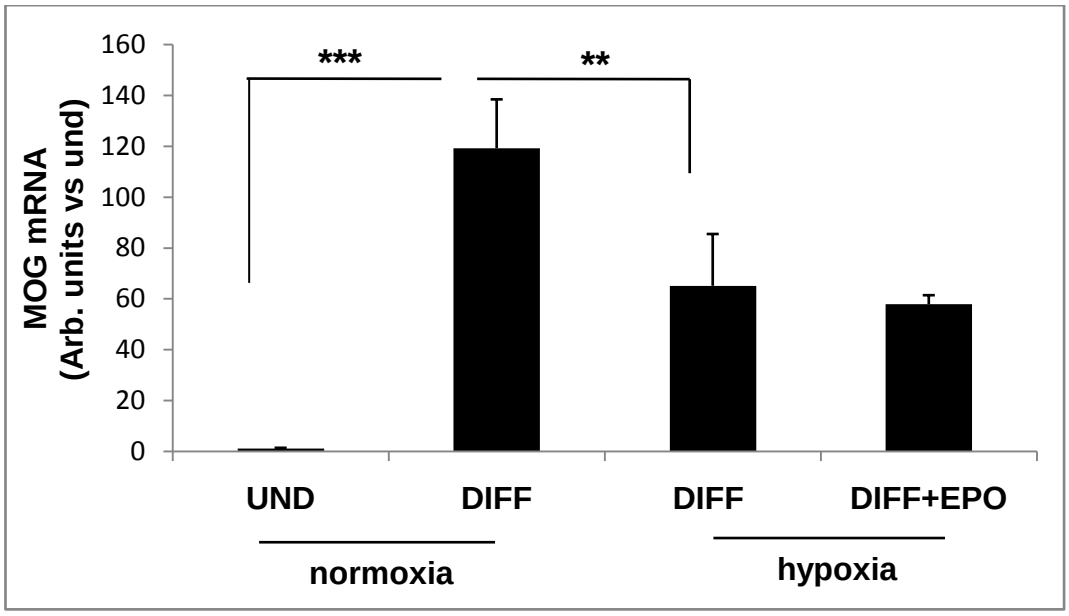


Figure 5.2: EPO does not induce MOG gene expression when EPOR is induced in CG4 WT cells by hypoxia. Cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates and the day after they were differentiated in normoxic or hypoxic condition (1% O2). Cells in hypoxia were also treated with EPO. The experiment was stopped after 5 days of differentiation. MOG gene expression was analysed by qPCR using HPRT1 as housekeeping gene. Results are the average of three samples analysed in duplicate. They are expressed as arbitrary units ± SD *vs* undifferentiated (UND) cells in normoxia. **P<0.01; ***P<0.001. The results suggest that the induction of EPOR in hypoxic conditions is not enough to mediate EPO effect on MOG induction.

5.3 Cloning of the CG4 WT cell line by limiting dilution

We have shown that it was not possible to induce EPOR in CG4 WT cells at a level sufficient to see an effect by EPO. For this reason, another approach was chosen and CG4 WT cells were cloned by limiting dilutions. In fact, since the expression of the receptor in the CG4 WT cell line was very low, we investigated whether it might be possible to isolate clones expressing different levels of EPOR and then study the effect of EPO in the ones with the highest expression.

A batch of 100 cells were distributed among three 96 well plates giving, on average, 0.3 cells/well. After a few days it was possible to see the first cells, almost 25 colonies in total. Some of them were discarded since clearly derived from more than one cell per well, leaving 13 clones that were grown and expanded in 24 well plates. Half of the cells of each new clone line were frozen and the other part expanded to collect cells for mRNA extraction.

EPOR gene expression was analysed in these clones by qPCR. It was found that clones expressed different levels of EPOR as shown in Fig. 5.3. However, the clone expressing the highest amount of EPOR (WT3) had a very low expression compared to the amount of EPO receptor in the transfected line (EPOR expression in CG4EPOR was approximately 500 fold greater than in the CG4 clone WT3). Unfortunately, clone WT3 could not be used for further investigations since the cells were of poor quality. Therefore, clone WT1, which expressed levels of EPOR lower than WT3 but still higher than WT cells, was used to investigate the effect of EPO on myelin gene induction and to determine whether this low expression of the receptor was sufficient to mediate myelin gene induction by EPO. For the experiment, 3×10^4 cells/well were plated in 24 well plates with GM and they were differentiated the day after with the DM. Cells were treated with EPO 80 ng/ml during 6 days of differentiation. The cDNA was analysed by qPCR for MOG gene expression by Taqman assay.

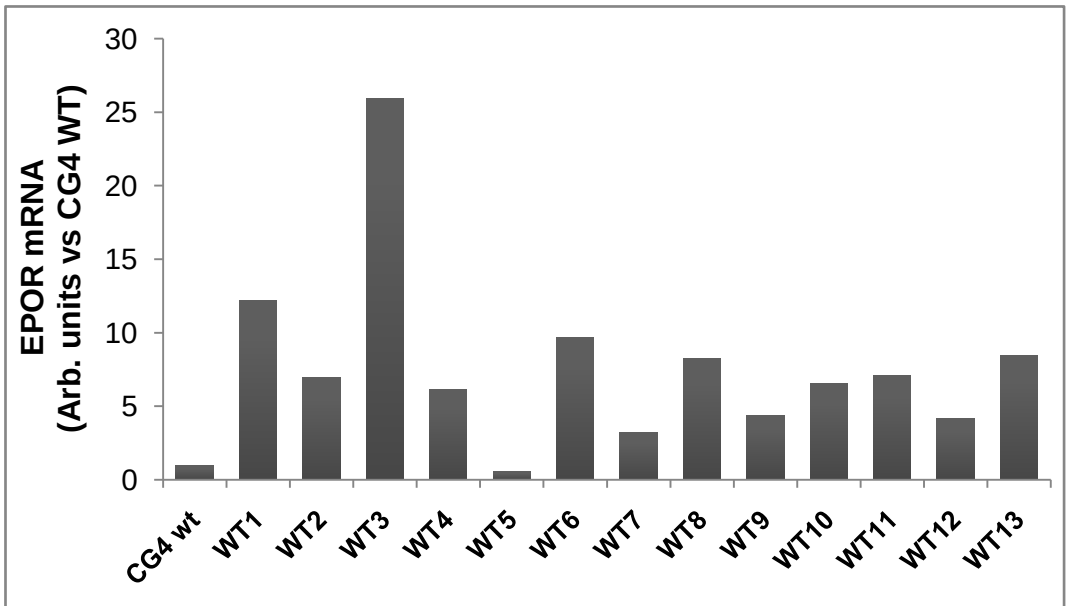


Figure 5.3: Clones isolated by limiting dilution from the CG4 WT cell line. 100 cells were distributed among three 96 well plates to have 0.3 cells/well. After one week, 13 clones were expanded and plated again in 24 well plates. The cDNA was analysed by qPCR for EPOR expression, using HPRT1 as housekeeping gene. Results are the average of single samples analysed in duplicate and are expressed as arbitrary units vs the CG4 WT cell line.

As shown in Fig. 5.4, oligodendrocytes were at a mature stage after 6 days of differentiation, since the expression of MOG was much higher than in the undifferentiated cells (11.8 ± 1.7 DIFF cells *vs* 1 ± 0.01 UND cells). EPO did not have any effect on MOG gene induction in these cells (13.8 ± 0.5 DIFF + EPO *vs* 11.8 ± 1.7 DIFF cells) (Fig. 5.4).

In conclusion, the small expression of EPOR obtained by cloning the CG4 WT cell line by limiting dilution was not sufficient to mediate the EPO effect on induction of myelin genes.

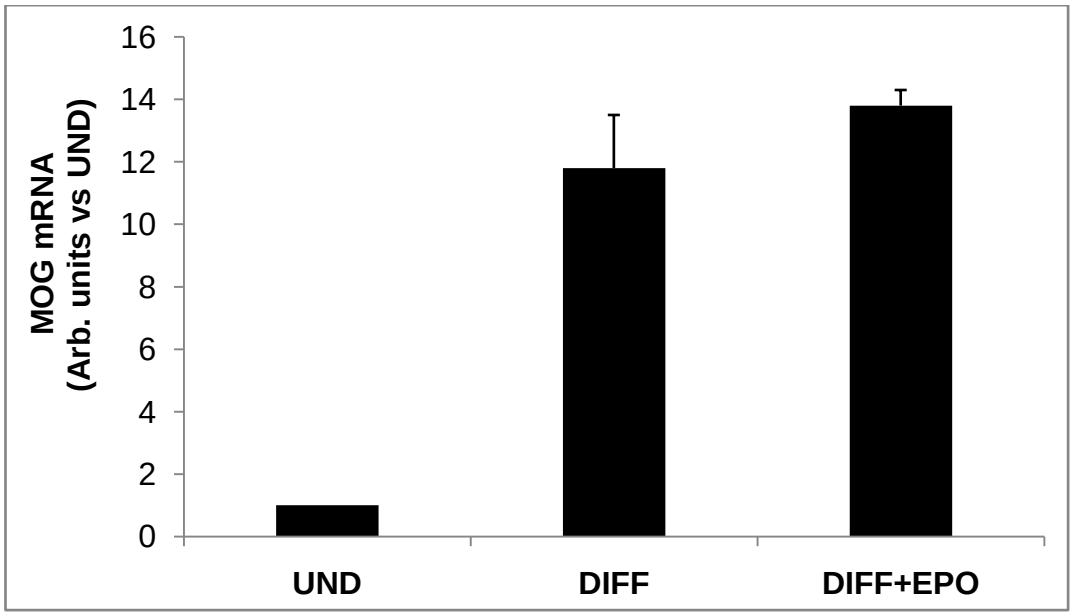


Figure 5.4: EPO did not induce MBP in CG4 WT clone WT1 cells. 3 X 10^4 cells/well were plated in 24 well plates. Cells were treated with EPO 80 ng/ml during 6 days of differentiation. The cDNA was analysed by qPCR for MOG gene expression by Taqman assay, using HPRT1 as housekeeping gene. Results are the average ± SD of three samples analysed in duplicate and expressed as arbitrary units *vs* undifferentiated cells.

5.4 Cloning of the CG4EPOR cell line by limiting dilution

So far, it has been demonstrated that EPO has an effect only in CG4EPOR, but no effect in CG4 WT cells. This result clearly suggests that EPOR is necessary to mediate the effect of EPO on myelin gene induction. However, EPO was shown to have no effect when trying to overexpress EPOR in CG4 WT cells, either by induction or cloning. One reason could be that EPOR expression in CG4 WT cells, although induced a little, was not enough to mediate the EPO effect. Moreover, another reason could be that the effect of EPO on CG4EPOR cells was not due to the overexpression of the receptor, but to an artefact caused by manipulation of these cells. To exclude this possibility, and to definitely show that the effect of EPO was mediated by EPOR, the CG4EPOR cells were cloned by limiting dilution. With this experiment it might be possible to obtain cell clones, manipulated in the same way, with a low expression of EPOR similar to the one of CG4 WT clones. The aim was to demonstrate that EPO no longer had an effect in these clones when the amount of receptor was dramatically decreased.

The cloning was done as described above for CG4 WT cells, with 21 clones expanded and analysed by qPCR for EPOR gene expression. Clones expressed different levels of EPOR, as reported in Fig. 5.5. Two clones were chosen for further investigations: clone R9, approximately only 12.5 times the expression of the receptor in clone WT1, and clone R15, about 775 times EPOR expression compared to clone WT1.

The effect of EPO on myelin gene induction was investigated in clones R9 and R15 and in the CG4EPOR cell line. MOG gene expression was analysed by qPCR after 6 days of differentiation. The analysis in clone R9 showed that there was only a small (1.7-fold) increase in induction of MOG when EPO was present (1.82 ± 0.5 DIFF+EPO *vs* 1.1 ± 0.1 DIFF; $P<0.05$) (Fig. 5.6). In clone R15 the induction by EPO was higher than in clone R9, with a 4.4-fold increase (3.95 ± 1.4 DIFF+EPO vs 0.9 ± 0.3 DIFF; $P<0.02$) (Fig. 5.6).

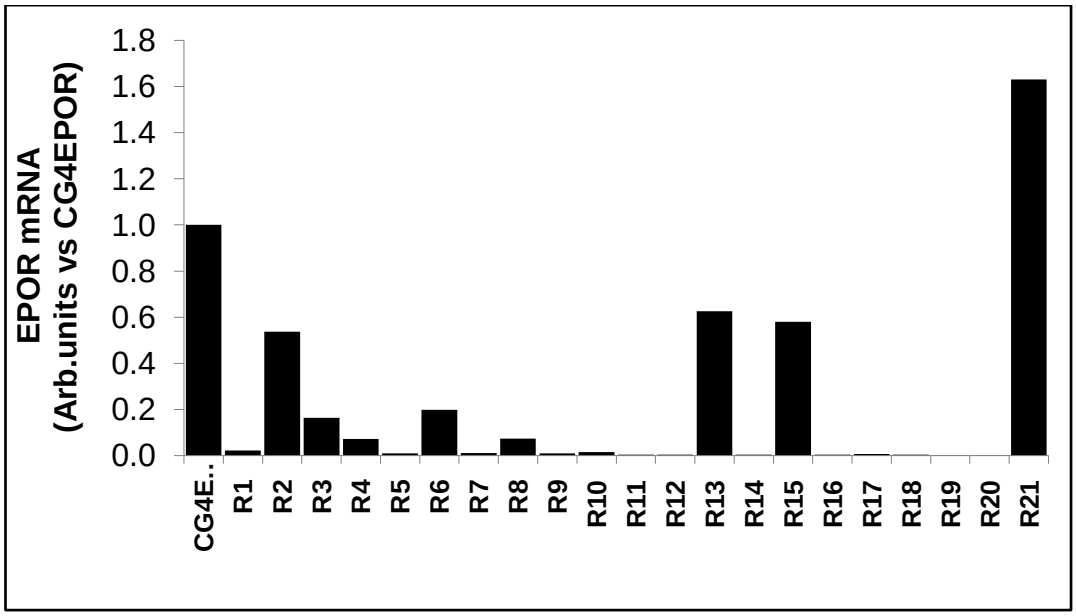


Figure 5.5: Clones isolated by limiting dilution from the CG4 EPOR cell line. 100 cells were distributed among three 96 well plates to have 0.3 cell/well. After one week, 21 clones were expanded and plated again in 24 well plates. When confluent, half of each cell line was frozen and half was harvested with TRIzol for RNA extraction and reverse transcription. The cDNA was analysed by qPCR for EPOR expression. Results are the average of single samples analysed in duplicate and are expressed as arbitrary units *vs* CG4EPOR cell line.

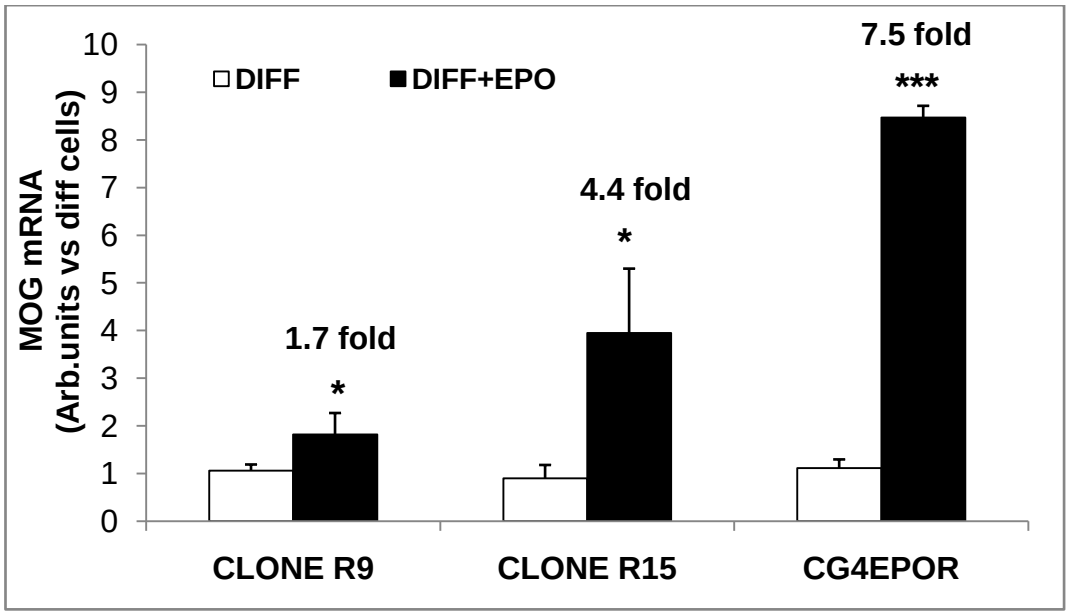


Figure 5.6: EPO induces MOG in a receptor-dependent manner. 3 X 10^4 cells/well were plated from clone R9, R15, and from the CG4EPOR cell line in 24 well plates. Cells were treated with EPO 80 ng/ml during 6 days of differentiation. The cDNA was analysed by qPCR for MOG gene expression. Results are the average ± SD of three samples analysed in duplicate and expressed as arbitrary units *vs* differentiated cells. *P<0.05; ***P<0.001.

In the CG4EPOR cell line, the induction of MOG was 7.5-fold greater in presence of EPO (8.5 ± 0.2 DIFF+EPO vs 1.1 ± 0.2 DIFF; P< 0.001) (Fig. 5.6). The conclusion of this experiment was that the effect of EPO on induction of MOG gene expression was lower when the amount of EPOR decreased, although all these cells were manipulated in the same way. This outcome supports the previous results and demonstrates that the EPO effect is mediated by EPOR.

The small effect of induction of myelin genes by EPO in clone R9 gives also an explanation of why EPO was not effective in all the experiments with CG4 WT cells and in clone WT1. In fact R9 expresses EPOR about 12.5 times more than WT1 and about 143 times more than CG4 WT cells (Table 5.1). So maybe the expression of EPOR in WT cells was not enough to mediate the EPO effect.

	Arbitrary units *vs* R9	Fold change *vs* R9
R9	1	1
R15	62	62
CG4 EPOR	107	107
CG4 WT	0.007	-142.9
WT1	0.08	-12.5

Table 5.1: Comparison of EPOR expression in different cell types.

5.5 Summary of chapter 5

The aim of this chapter was to demonstrate a correlation between the EPO effect on myelin gene induction and the expression of EPO receptor.
In the first part of this chapter, we tried to obtain CG4 WT cells expressing higher levels of EPOR, to demonstrate an effect of EPO on myelin genes in cells not genetically modified. Hypoxia (1% O_2) induced EPOR but no effect of EPO was observed. Afterwards, a cloning by limiting dilutions of the CG4 WT cell line was performed, to isolate clones expressing higher amounts of EPOR. The aim was to investigate the effect of EPO on myelin gene expression in the clone that expressed the highest amount of EPO receptor. Again, EPO did not induce MOG in this clone. It is possible that the absence of an EPO effect in the models used so far may be due to the very low expression of the receptor in these clones compared to that of the CG4 EPOR cells (which was 500-folds greater).
In the second part of this chapter, a cloning by limiting dilutions of the CG4 EPOR cell line was done. The goal of this experiment was to demonstrate that the effect of EPO was related to the expression of the receptor and not due to a cell manipulation: 21 clones, expressing different amounts of EPOR, were isolated. The induction of MOG by EPO was studied in two of these clones with a low and an intermediate expression of EPOR. The outcome was a lower induction of MOG by EPO in the clone expressing a lower amount of EPOR and a greater induction in the clone expressing a higher level of EPOR.
In conclusion, the results obtained in this chapter confirm that EPOR is required by EPO to mediate the induction of myelin genes.

Chapter 6: Study of intracellular pathways mediating EPO effect

In the last part of this study, pathways activated by EPO to induce myelin genes were investigated. In a previous work in our laboratory, it was found that the early growth response gene 2 (EGR2, also called Krox-20) was one of the main genes induced by EPO in an animal model of stroke obtained by middle cerebral artery occlusion (MCAO), in which EPO was protective. That result was confirmed *in vitro* in a line of neuroblastoma cells in which EPO induced EGR2 expression.

EGR2 is an early gene that is also specifically required in the peripheral nervous system to initiate and maintain myelination (Topilko et al., 1994). To date, its role in the central nervous system myelination is unknown and in experiments of more than 15 years ago it was not even detected in oligodendrocyte cells (Sock et al., 1997).

The hypothesis that EGR2 could be involved in mediation of EPO effect in oligodendrocyte cells was investigated in this chapter, starting to investigate the presence of EGR2 in the CNS and some possible correlation with its myelination.

Another pathway investigated, that might mediate EPO effect on myelination, was the PI3K/Akt/mTOR pathway, that was demonstrated to be involved in proliferation, migration and survival of OPCs (Ebner et al., 2000) (Gomez et al., 2011). In addition, mTOR mediated some effect of EPO like prevention of microglia apoptotic injury during oxidative stress (Shang et al., 2012).

6.1 Analysis of EGR2 expression in CG4EPOR cells and induction by EPO

EGR2 was studied in the CG4 cell line to see whether it was expressed in these cells and modulated by EPO. CG4EPOR cells were plated at a concentration of 3 X 10^4 cells/well in 24 well plates. When sub-confluent, cells were differentiated and after 4 hours were treated with EPO 80 ng/ml for 1 hour. The cDNA was analysed by qPCR for EGR2 gene expression. A basal level of EGR2 expression was found in CG4EPOR cells and this expression was higher in undifferentiated cells than in differentiated cells, where the amount decreased of more than 10 times (1.02 ± 0.2 undifferentiated cells vs 0.1 ± 0.02 differentiated cells) (Fig. 6.1). One hour of EPO treatment dramatically induced EGR2 expression of about 18 folds compared to the differentiated cells (1.8 ± 0.6 differentiated plus EPO *vs* 0.1 ± 0.02 differentiated cells; $P< 0.05$ by Student's *t* test) (Fig. 6.1).
The outcome of the experiment was that EGR2 was surprisingly expressed in oligodendrocyte precursor cells, decreased during differentiation and increased by 1 hour of EPO treatment. This result leads to the hypothesis that EGR2 could be involved in mediation of EPO effect on myelin gene induction. Therefore, this hypothesis was further investigated.

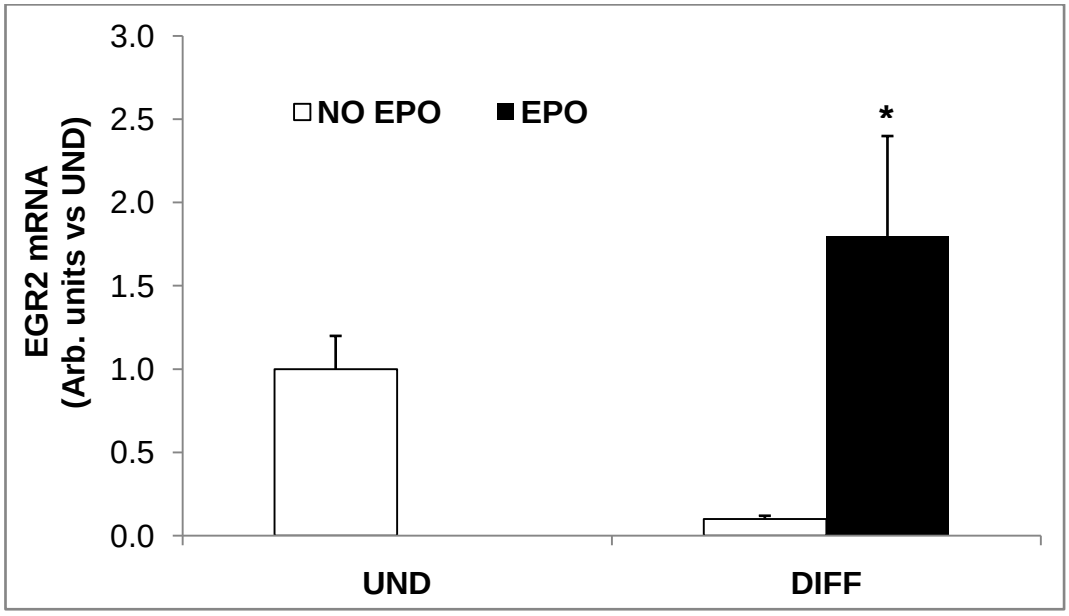


Figure 6.1: EPO induced EGR2 gene expression in CG4EPOR cells after one hour of treatment. 3 X 10^4 cells/well were plated in 24 well plates in GM. After one day cells were differentiated with the DM. After 4 hours cells were treated with EPO 80 ng/ml for one hour and then the experiment was stopped. The cDNA was analysed by qPCR for EGR2 gene expression that was expressed as arbitrary units *vs* undifferentiated cells. Results are the average ± SD of three samples analysed in duplicate. *P<0.05 *vs* undifferentiated cells. The experiment is representative of three independent experiments.

6.2 EGR2 induction by EPO in CG4 WT cells

Before further investigate whether EPO might increase myelin gene expression through induction of EGR2, this early response gene induction was studied in CG4 WT cells. In fact, if EGR2 is required to mediate MOG induction, it should not be induced by EPO in CG4 WT cells that are not responsive to EPO in terms of MOG induction. 3 X 10^4 cells/well were plated in 24 well plates and left in GM for one day. Afterwards, cells were differentiated with DM for 4 hours and treated for one hour with EPO 80 ng/ml. The same scheme described in 6.1 was used to be able to compare EPO-induced EGR2 in CG4 WT and EPOR cells. EGR2 gene expression was measured by qPCR. No significant induction of EGR2 by EPO was found in differentiated cells (0.1 ± 0.01 differentiated cells *vs* 0.2 ± 0.03 differentiated plus EPO) (Fig. 6.2).

The result showed that EPO receptor expression was required to mediate EGR2 induction by EPO, like it was necessary to mediate myelin gene induction.

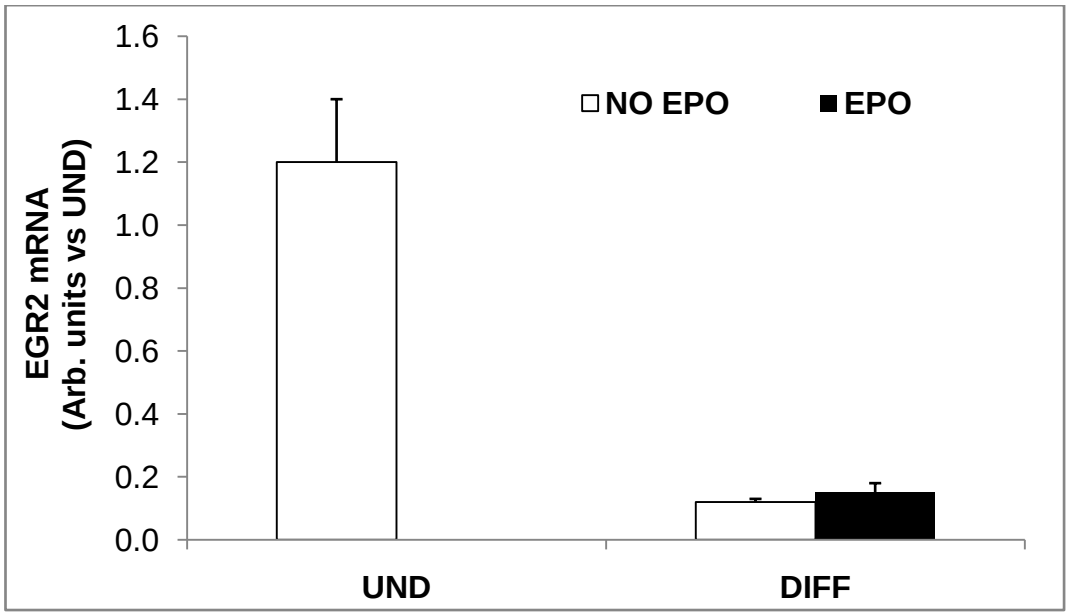


Figure 6.2: EGR2 is not induced by EPO in CG4 WT cells. Cells were plated at 3 X 10^4 cells/well in 24 well plates in GM. The day after cells were differentiated in DM and after 4 hours were treated with EPO 80 ng/ml for 1 hour. EGR2 gene expression was analysed by qPCR. Results are the average of three samples analysed in duplicate and are expressed as arbitrary unit ± SD *vs* undifferentiated cells.

6.3 Set up of EGR2 siRNA transfection

The hypothesis that myelin genes might be induced by EPO through induction of EGR2 was studied silencing EGR2 gene expression by transfection with an siRNA. The transfection in CG4EPOR cells was set up by several pilot experiments to find the right model of transfection to use and times of treatment. A first experiment was run to choose between two methods of transfection: forward and reverse.

For the forward method, 35 X 10^3 cells/well in 24 well plates were suspended in a final volume of 500 μl in GM. The day after, 100 μl/well of transfection solution were added. The transfection agent was prepared by mixing the EGR2 siRNA (30 nM) in Optimem with Lipofectamine and then leaving the mix for 15 minutes in incubation. For the reverse method, the same amount of cells was plated and immediately treated with the transfection solution. For both schemes the time point was at 36 hours, as suggested in the manufacturer instructions.

The yield of transfection of the two methods was exactly the same (0.3 ± 0.03 reverse transfection *vs* 0.9 ± 0.1 control and 0.3 ± 0.1 forward transfection vs 1 ± 0.1 control) (Fig. 6.3). The forward method was chosen for the following experiments because the transfection could be done at the time of cell differentiation.

The next experiment was focus on investigating the time at which the silencing of the gene was already effective and for how long this effect could last. The first time point analysed was at 24 hours from the transfection ± 1 hour of EPO treatment, as control to check that the EPO effect was blocked and the transfection effective. The other time points were at 2 and 4 days of differentiation after transfection ± EPO treatment, times at which induction of myelin genes by EPO could be studied. No later time points were analysed since the siRNA could not be effective longer than 4 days.

Finally, the experiment was done plating 35 X 10^3 cells/well in 24 well plates. The day after, cells were differentiated with DM and at the same time they were treated with the EGR2 siRNA and stopped after 1, 2 and 4 days. Cells were also treated with EPO during differentiation.

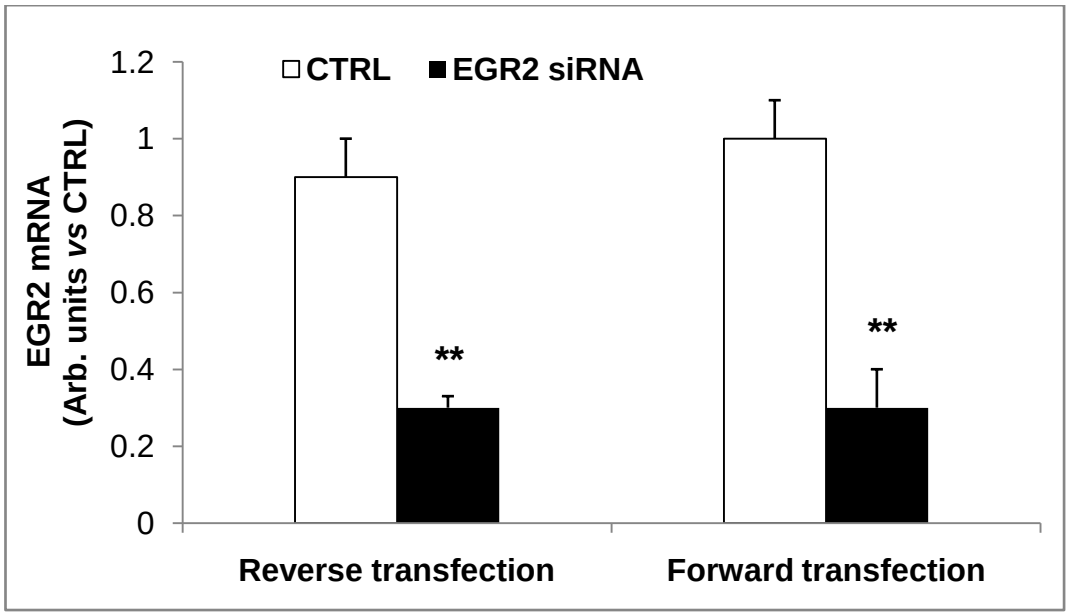


Figure 6.3: Comparison of the efficiency of two transfection methods. Reverse transfection: cells were plated and treated, at the same time, with a transfection mix composed of EGR2 siRNA (30nM) in Optimem plus Lipofectamine. Forward transfection: cells were plated and the following day treated with the transfection mix at the same as for the reverse transfection. This experiment was stopped after 36 hours from the transfection. Samples were analysed by qPCR for EGR2 gene expression. Results are the average ± SD of three samples analysed in duplicate and expressed as arbitrary units *vs* each CTRL. **P<0.01 *vs* CTRL.

The result showed that the transfection was already effective after 24 hours, blocking EGR2 induction by EPO (13.25 ± 0.7 without siRNA *vs* 2.7 ± 0.2 with the siRNA) (Fig. 6.4 A) and still silencing EGR2 after 4 days (0.4 ± 0.02 without siRNA *vs* 0.12 ± 0.04 with the siRNA) (Fig. 6.4 B). Obviously, the siRNA was silencing also at the intermediate time point of 2 days (data not shown).

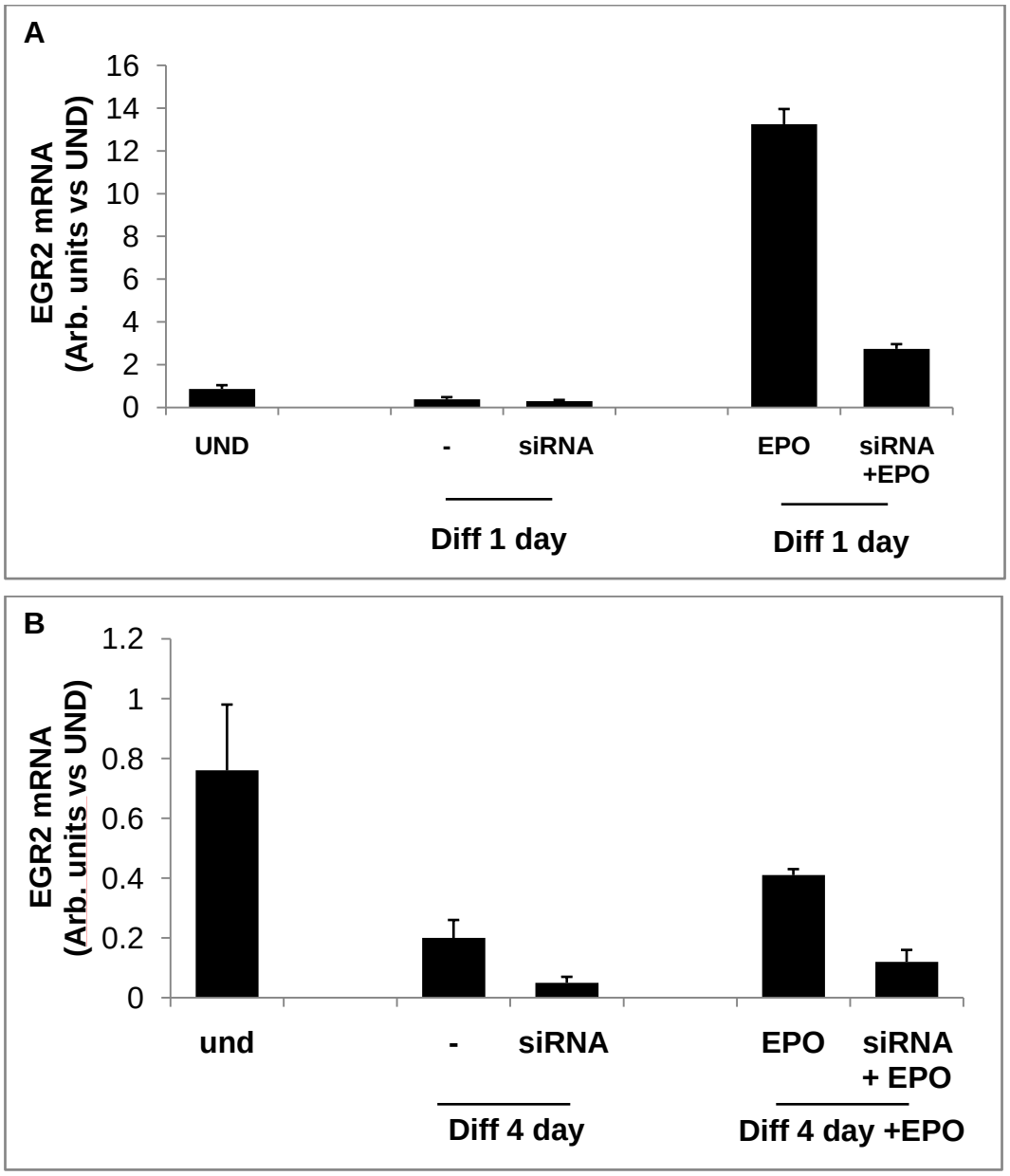


Figure 6.4: EGR2 siRNA knocked down EGR2 gene after 24 hours and 4 days. CG4EPOR cells were plated at a concentration of 35 X 10^3 cells/well in GM without pen/strep. The day after cells were differentiated with DM and at the same time they were treated with the EGR2 siRNA and stopped after 1 (A) and 4 (B) days. EPO treatments were also done during differentiation. Samples were analysed by qPCR for EGR2. Results are the average ± SD of three samples analysed in duplicate and are expressed as arbitrary units *vs* undifferentiated cells.

6.4 Study of the EGR2 pathway that could mediate EPO induction of myelin genes

According to the result of preliminary experiments, the scheme used to study myelin gene expression was: transfection at the day of differentiation, treatment with EPO after 24 hours when the gene was already knocked down and end of the experiment after 4 days when EGR2 was still blocked by the siRNA (summary in Fig. 6.5). The aim of this experiment was to investigate whether EPO induces myelin genes trough EGR2 induction. Therefore, by silencing EGR2 gene expression a lower induction of myelin genes by EPO should be found.

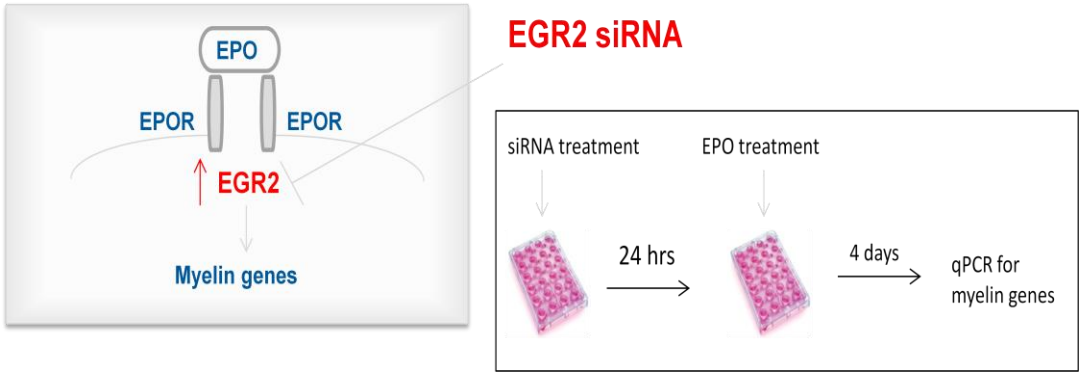


Figure 6.5: Scheme of the EGR2 siRNA transfection experiment.

Cells were plated at 3 X 10^4 cells/ml in 24 well plates in GM. After one day cells were differentiated and transfected with the EGR2 siRNA. EPO treatment was started after 24 hours from transfection. The DM was changed at day 3 and a new EPO treatment was done. At day 4, cells were harvested with TRIzol and the RNA extracted. The cDNA was analysed by qPCR for MBP gene expression. The result was that EPO still increased the expression of MBP in the group treated with EGR2 siRNA, showing that EGR2 is not a pathway for EPO myelin gene induction (27.2 ± 2.1 siRNA+EPO *vs* 14.8 ± 1.8 EPO alone; P<0.01) (Fig. 6.6).

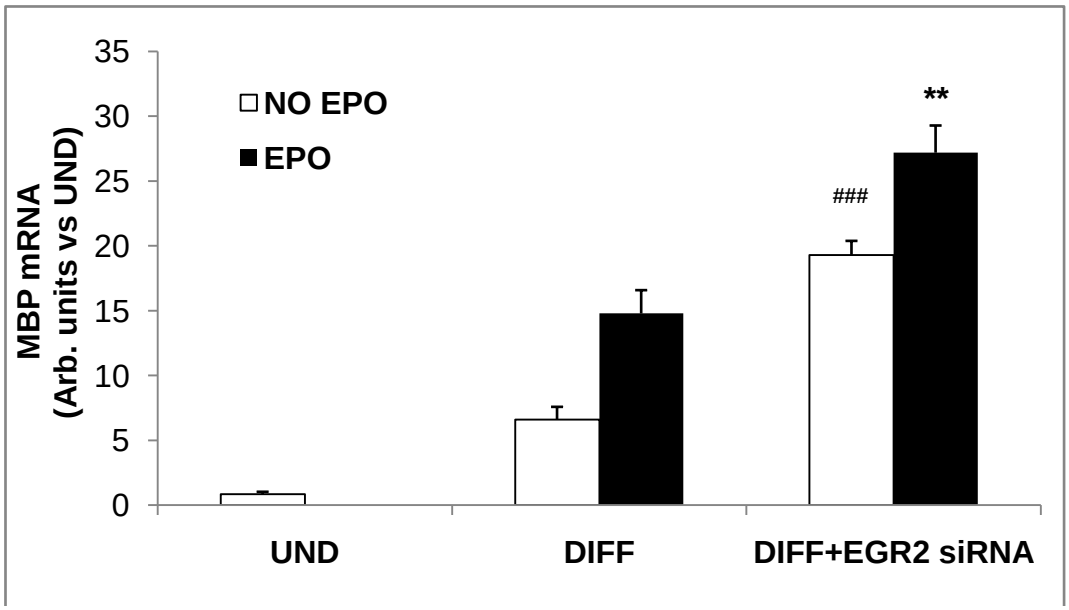


Figure 6.6: EPO does not act through EGR2 to induce MBP gene expression. 30,000 cells/ml were plated in GM and differentiated the day after with the DM. At the time of differentiation also the transfection with the siRNA for EGR2 was done to knock down EGR2 gene expression. EPO treatment 80 ng/ml was done at day 1 (24 hours after the transfection) and at day 3. Samples were collected with TRIzol at day 4. The resulting cDNA was analysed by qPCR for MBP gene expression. Results are the average of three samples analysed in duplicate and are expressed as arbitrary units ± SD *vs* UND. **P<0.01 *vs* EPO DIFF; $^{###}$P<0.001 *vs* NO EPO DIFF.

Even if EGR2 did not mediate EPO induction of myelin genes, an interesting result came out from this experiment. The group treated with the siRNA alone, without EPO, showed an higher induction of MBP compared to the control (19.3 ± 1.1 siRNA *vs* 6.6 ± 1 no siRNA; P<0.001) (Fig. 6.6).

For this reason the experiment was repeated using a negative siRNA control, to investigate whether the induction of MBP was due to the EGR2 silencing or to the transfection with an siRNA. The same experiment described above was repeated transfecting cells with the EGR2 siRNA and with a control siRNA. MBP was analysed by qPCR after 4 days of differentiation.

The result showed a higher expression of MBP by silencing EGR2 (22.3 ± 3.5 EGR2 siRNA *vs* 11.1 ± 2.3 differentiated control; P<0.01) (Fig. 6.7). In the group with the control siRNA the induction of MBP was exactly the same as in the group without siRNA (11.6 ± 2 CTRL siRNA vs 11.1 ± 2.3 differentiated control) (Fig. 6.7).

The induction of MBP by EPO was confirmed again in the group transfected with the EGR2 siRNA and it was also confirmed that EPO still induced MBP when EGR2 was silenced (33.3 ± 2.4 EGR2 siRNA + EPO *vs* 19.6 ± 3.8 CTRL + EPO).

The conclusion of these experiments was that EPO did not induce myelin gene expression through EGR2 induction but, following the silencing of EGR2 there was an increase in MBP gene expression. The outcome of this experiment could be the hypothesis that EGR2 inhibition might increase oligodendrocytes differentiation. Further investigations in primary cells, would be useful to understand whether there is an involvement of EGR2 in myelination in the CNS.

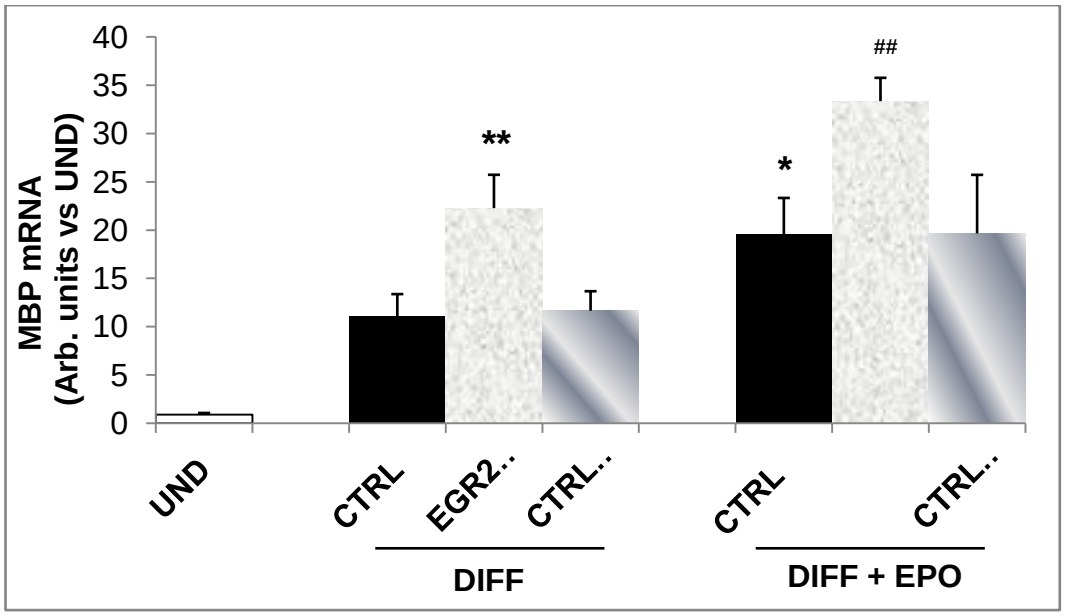


Figure 6.7: The EGR2 knock-down increases MBP gene expression. Cells were plated at 3 X 10^4 cells/well in 24 well plates. After 1 day cells were differentiated and transfected. EPO treatment was also done during the 4 days of differentiation, starting at day 1. Several groups were considered: undifferentiated and differentiated plus EGR2 siRNA or control siRNA. These groups were treated with and without EPO 80 ng/ml. The cDNA was analysed by qPCR for MBP gene expression. Results are the average of three samples analysed in duplicate and are expressed as arbitrary unit ± SD vs UND. **P < 0.01 vs CTRL DIFF; *P < 0.05 vs CTRL DIFF; $^{\#\#}$P < 0.01 vs CTRL DIFF+EPO.

6.5 EGR2 expression in primary oligodendrocytes and EPO effect

To date, EGR2 was found expressed at basal levels in oligodendrocyte precursor cells and decreased during differentiation. Since previous studies only found that EGR2 was not expressed in primary oligodendrocytes cells (Sock et al., 1997) we wanted to confirm the data obtained in the cell line also in the primary cells. From the collaboration with Mark Kotter laboratory, we looked at EGR2 expression in rat primary undifferentiated precursor cells and during differentiation at different time points. Cells were also treated with EPO 80 ng/ml to confirm the induction of EGR2 by EPO.

Primary oligodendrocytes precursor cells showed a basal level of EGR2 expression, as seen by qPCR. This expression decreased during differentiation, showing a trend similar to that of the CG4 cell line.

No effect of induction of EGR2 by EPO was seen at any time point analysed (Fig. 6.8).

Maybe, EPOR is required by EPO to induce EGR2 as for myelin gene induction. Therefore, the low expression of EPOR in primary cells does not allow to have an induction of EGR2 gene expression by EPO.

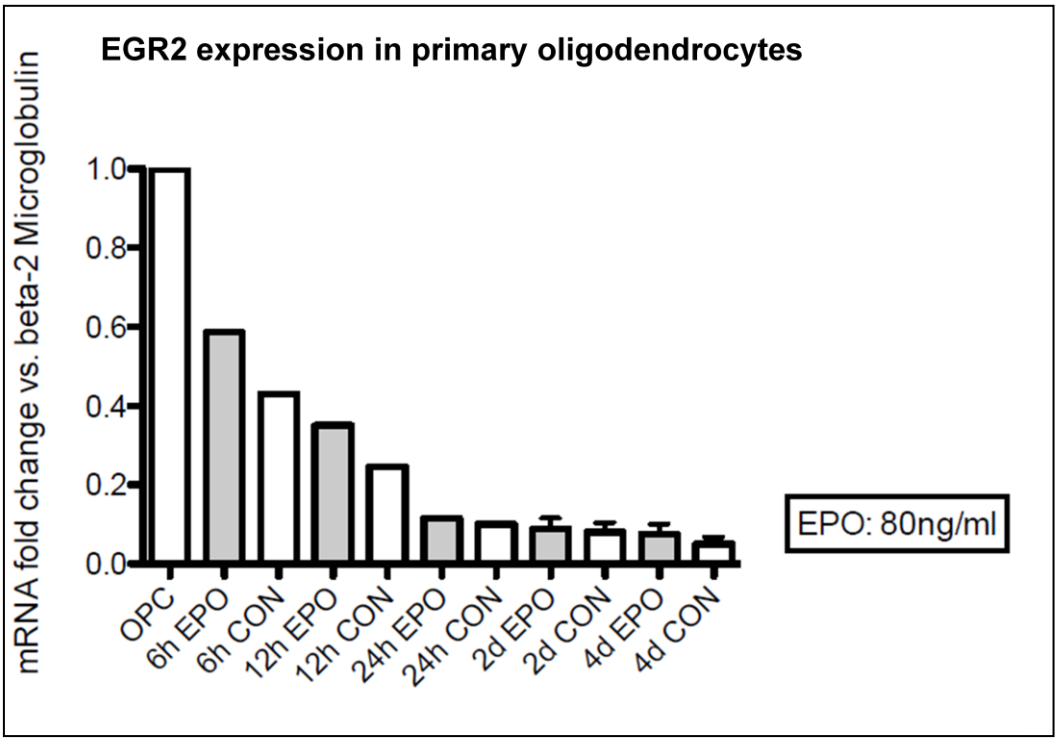


Figure 6.8: EGR2 is expressed in oligodendrocyte cells and EPO does not have an effect on induction of EGR2 expression. Primary oligodendrocyte precursor cells and oligodendrocyte cells with EPO or control (CON) at different times during differentiation were analysed by qPCR for EGR2 expression. There was a basal expression of EGR2 in precursor cells that decreased during differentiation. No effect of EGR2 induction by EPO was found.

6.6 Study of mTOR pathway that could mediate EPO induction of myelin genes

The second hypothesis was that EPO might induce oligodendrocytes differentiation, seen by induction of myelin genes, through PI3K/Akt/mTOR pathways.

Several studies correlate PI3K/Akt pathway with proliferation, migration or survival of OPCs (Ebner et al., 2000) (Cui and Almazan, 2007) (Flores et al., 2008). In addition, the mammalian target of rapamicin (mTOR), downstream signal of PI3K/Akt pathways, was shown to be essential for oligodendrocytes differentiation (Tyler et al., 2009). Moreover, it was specifically demonstrated that inhibition of mTOR attenuates the progression of oligodendrocyte progenitor cells to the mature stage by inhibiting the expression of myelin proteins (MBP, MOG) (Guardiola-Diaz et al., 2012). Effects of EPO mediated by the mTOR pathway were also demonstrated in the last years in bone formation (Kim et al., 2012), prevention of β-amyloid degeneration and inflammation in microglia (Shang et al., 2012) (Shang et al., 2011) and in retinal progenitor cells survival (Sanghera et al., 2011), showing a possible correlation between EPO and mTOR.

To study this mechanism, the KU 0063794, a specific inhibitor of mTOR and rapamicin analogue, was used. KU 0063794 inhibits mTOR in 1 hour. The scheme of the planned experiment is show below (Fig.6.9).

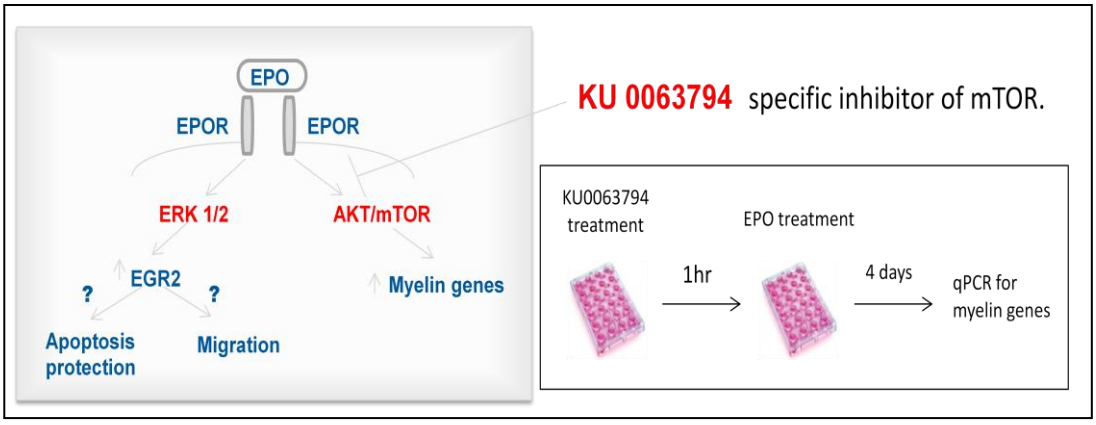


Figure 6.9: Scheme of the experiment to inhibit mTOR.

As preliminary, a viability assay to test its toxicity in these cells was done. Cells were plated in a pre-coated 96 well plate at a concentration of 5,000 cells/well and differentiated after one day. Cells were treated with KU 0063794 at the doses of: 10 μM, 1 μM and 0.1 μM. Treatments were repeated during 4 days of differentiation at every change of medium. The last day the CTB was added into the wells and the fluorescence was read after 4 hours by microplate reader using excitation 530/25 nm and emission 590/40 nm filters (Fig. 6.10). KU 0063794 was toxic at all doses used. The dose of 0.1 μM was slightly but still significantly toxic; therefore this compound could not be used for the experiment we would have liked to do.

A possible way to inhibit the mTOR pathway could be the same used for EGR2: the use of an siRNA to knock down mTOR gene expression. Unfortunately, it was not possible to carry on this second part of the study but it could be a future work to investigate the mechanism of action of EPO in myelination.

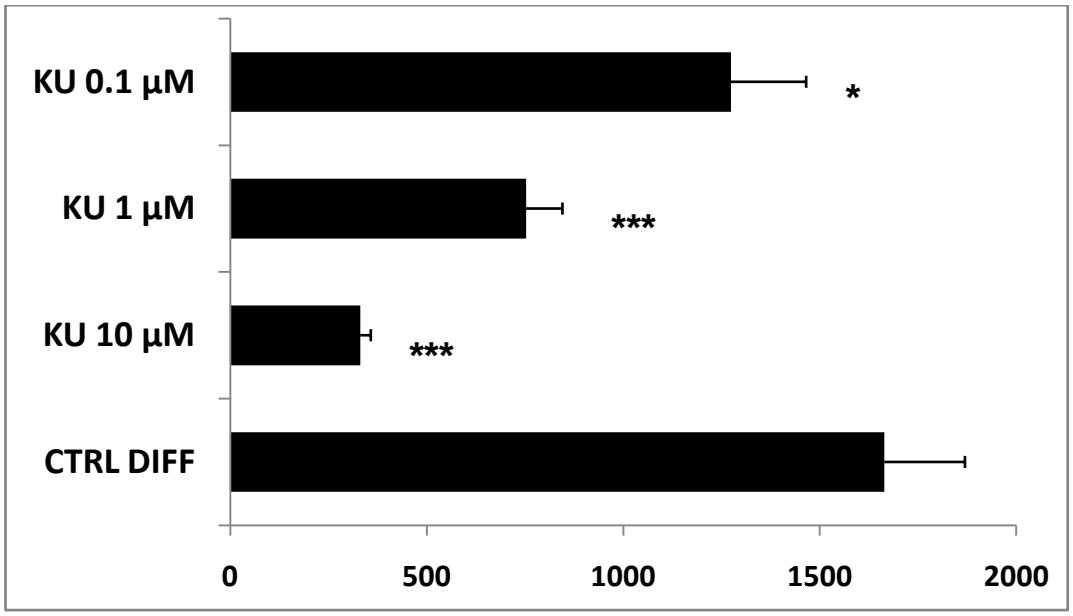


Figure 6.10: Cell Titer Blue (CTB) assay to test cell viability. 5 X 10^3 cells/ml were plated in 96 well plates in GM. The day after, cells were differentiated and treated with KU 0063794 at different doses. The treatment was repeated for 4 days of differentiation. At day 4, CTB was added and the cell viability assessed by fluorescence after 4 hours. Results are the average ±SD of 4 samples for each group. *P<0.05; ***P<0.001 by Student's *t* test.

6.7 Summary of chapter 6

The aim of this chapter was to investigate the intracellular pathways activated by EPO to mediate the induction of myelin gene expression.
The first focus was on EGR2 induction by EPO. In CG4EPOR cells, one hour of EPO treatment induced EGR2 of about 33 folds after 4 hours of differentiation.
The hypothesis that the induction of myelin genes by EPO could be mediated by the increase of EGR2 expression was further investigated by using an siRNA for EGR2. EGR2 gene was silenced by the siRNA starting one day after the treatment and for 4 days. EPO still induced MOG and MBP gene expression when EGR2 was silenced; therefore this effect was not mediated by EGR2.
Surprisingly, the siRNA for EGR2 induced MOG and MBP gene expression as well, leading to the hypothesis that EGR2 inhibition could induce myelin genes.
Another pathway we tried to investigate was through mTOR, using a rapamicin analogue, the KU 0063794. Unfortunately, this compound was toxic for CG4 cells making impossible its use for further experiments.

Chapter 7: *In vivo* model of demyelination: CUPRIZONE

In the previous *in vitro* experiments, EPO showed a good effect on oligodendrocytes differentiation as seen by induction of myelin gene expression. Therefore, an *in vivo* model was also set up to confirm the myelin gene induction effect of EPO and maybe its role on myelination. EPO already showed some effects on promotion of oligodendrogenesis and myelin repair in some *in vivo* models of disease and could lead to recovery. In this regard, in a rat model of focal cerebral ischemia EPO increased the number of OPCs, the generation of new myelinating oligodendrocytes and the number of myelinated axons (Zhang et al., 2010); in a neonatal hypoxic/ischemic rat model EPO increased proliferation and maturation of OPCs and reduced white matter injury at a cellular level as seen using myelin basic protein (MBP) as marker of myelination (Iwai et al., 2010) (Yamada et al., 2011); finally, in a model of demyelination induced by lysolecithin it was shown that EPO induced myelin repair (Cho et al., 2012).

The model of cuprizone-induced demyelination was set up and the effect of EPO in this model was studied. Like in the *in vitro* work, myelin gene expression was measured to quantify the process of demyelination and remyelination, as also reported by others (Morell et al., 1998) (Jurevics et al., 2002). Cuprizone is a copper chelator able to induce central demyelination without damage to other cells in the central nervous system (CNS) other than oligodendrocytes and without systemic inflammation. This model was chosen mainly because it is a simple model, in which demyelination is due only to a damage to oligodendrocytes, without potentially confounding factors like T-cell mediated inflammation in experimental autoimmune encephalomyelitis (EAE), and therefore good to study a direct effect of EPO on myelination.

7.1 Set up of the cuprizone model.

Cuprizone (bis cyclohexanone oxaldihydrazone) is a selective copper chelating agent that damages oligodendrocytes directly, leading to their death. This demyelinating model needed to be set up, since never used in the lab before.
A first experiment was done with 46 C57/BL6 female mice, 7-8 weeks old and weighing about 20 grams each. Mice were fed with cuprizone (0.2%, Sigma-Aldrich; diet prepared from Special Diets Services) or normal chow for 3 or 5 weeks. Because spontaneous remyelination happens when the diet is suspended, an additional time point at 5 days post-cuprizone administration was also assessed to study the trend of myelin gene expression during the recovery phase of the disease. In the same experiment, as pilot experiment, some mice were also treated with EPO 50 μg/kg. The groups assessed were:

6 mice CONTROL 6 mice CUPRIZONE 6 mice CUPRIZONE + EPO	**3 weeks**
6 mice CONTROL 6 mice CUPRIZONE 6 mice CUPRIZONE + EPO	**3 weeks cuprizone + 5 days normal chow**
5 mice CONTROL 5 mice CUPRIZONE	**5 weeks cuprizone**

The time points were chosen according to Morrel et al., who reported that the low pick of myelin gene expression was observed at 3 weeks even if the highest induction of demyelination induced by cuprizone was observed at 5-6 weeks from the beginning of the diet. At 6 weeks the myelin gene

expression was increased again to values comparable to control mice (Morell et al., 1998).

The aim of this first experiment was to confirm the decrease of myelin oligodendrocyte glycoprotein (MOG) at three weeks of cuprizone diet, already reported (Morell et al., 1998), and whether EPO inhibited this decrease. In addition, the experiment aimed to investigate the number of normal chow days needed to reverse the change in MOG gene expression to the control values and look at the effect of EPO on this recovery. Finally, MOG gene expression by cuprizone insult at 5 weeks of treatment was also studied as Morrel stated that at this time point maximum demyelination was experienced (Morell et al., 1998).

At the indicated time points, mice were sacrificed and the region between bregma and lambda, including the corpus callosum, was frozen in liquid nitrogen and stored at -80ºC. RNA was then extracted and retro-transcripted for qPCR analysis. MOG gene expression was assayed by qPCR. Using this model, MOG expression was decreased 5 folds compared to the control group after 3 weeks of cuprizone diet (arbitrary units ±SD, 0.2 ± 0.1 cuprizone *vs* 1 ± 0.2 control group); with a significance of $P< 0.001$ obtained using a Student's *t* test (Fig. 7.1). After 5 days of normal chow the values were back to normal (1 ± 0.1 cuprizone *vs* 1 ± 0.2 control group) (Fig. 7.1). In the group treated for 5 weeks with a cuprizone diet, the level of MOG was not significantly different from that of the 3 weeks group, but still significantly lower than that observed in the control group (0.2 ± 0.1 at 3 weeks *vs* 0.3 ± 0.1 at 5 weeks; $P< 0.001$) (Fig. 7.1). The results obtained in this first pilot experiment confirmed in part Morell work, i.e. the MOG gene expression dramatically decreased after 3 weeks of cuprizone diet (Morell et al., 1998). However, at the 5 weeks time point, MOG was still lower than control levels in this model, implying that the 5 weeks time point could be used to study demyelination by analysing myelin gene expression. After 5 days of normal chow MOG expression was back to the control values.

There was no effect of EPO on MOG gene expression at both time points of 3 weeks and 3 weeks plus 5 days of normal chow (3 weeks: 0.2 ± 0.07 cuprizone vs 0.2 ± 0.1 cuprizone + EPO; 3 weeks+5 days: 1 ± 0.1

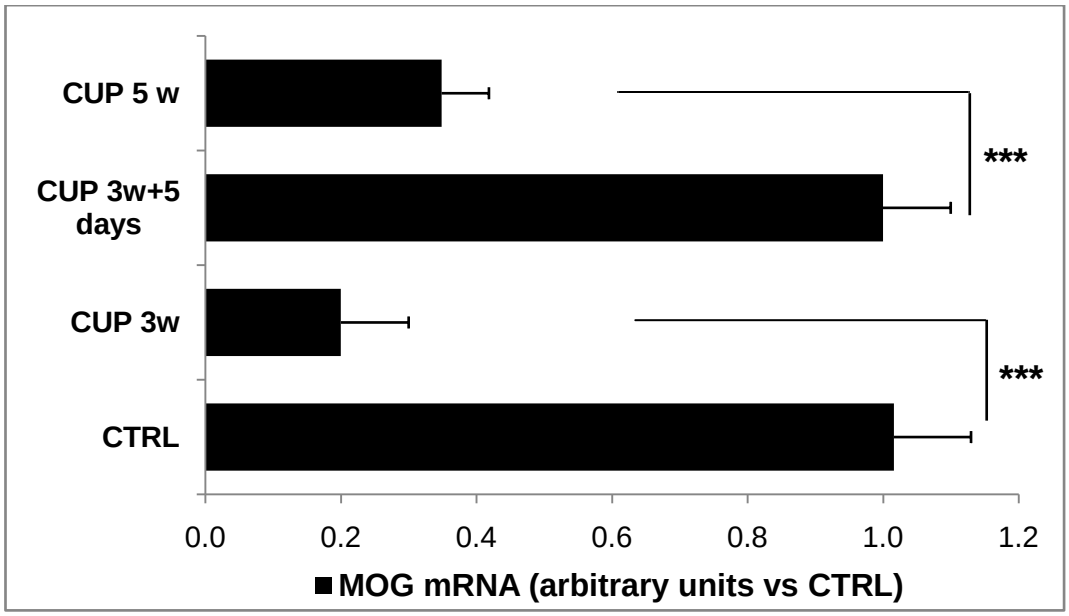


Figure 7.1: MOG gene expression trend in the demyelination model induced by cuprizone. C57/BL6 female mice from Charles River, 7-8 weeks old, were used. Mice were fed with 0.2% cuprizone diet for 3 weeks (n=6), 5 weeks (n=5) or 3 weeks plus 5 days of normal chow as recovery scheme (n=6); 6 mice were used as control. Mice were sacrificed at the indicated time points. MOG gene expression was analysed by qPCR and expressed as arbitrary units *vs* control. MOG expression was decreased 5 times compared to the control group after 3 weeks of cuprizone diet. Results are the average ± SD of samples analysed in duplicate; ***P<0.001 by Student's *t* test.

cuprizone vs 1.3 ± 0.1 cuprizone+EPO) (Fig. 7.2). However, 5 days of normal chow was a too long time point to study EPO effect in the remyelination phase, since there was already a complete recovery of MOG gene expression.

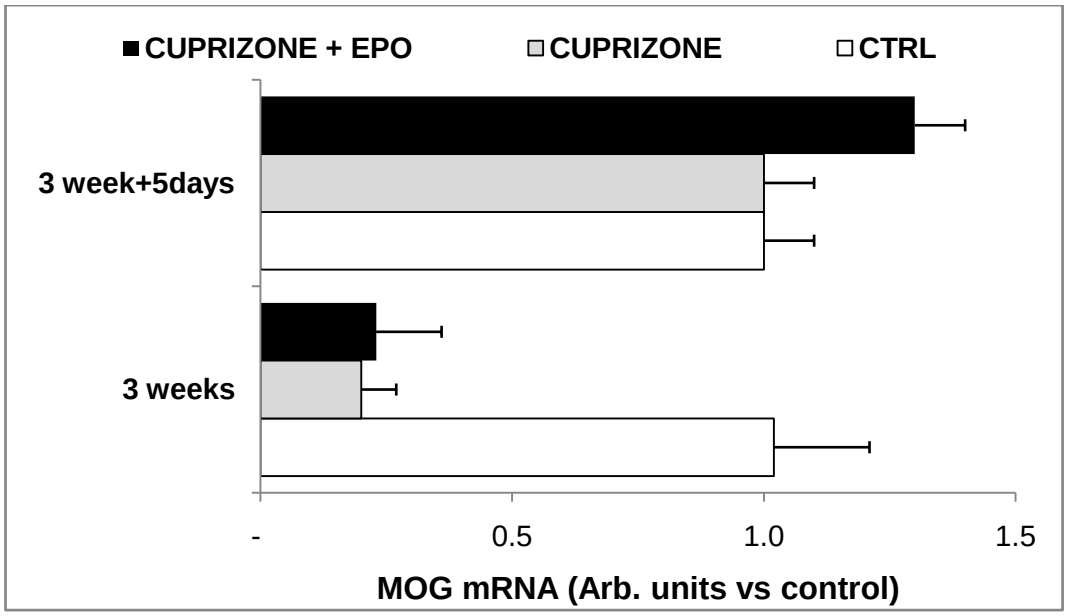


Figure 7.2: EPO did not have any effect on the cuprizone model at 3 weeks and 3 weeks plus 5 days of recovery. Mice were administered 0.2% cuprizone diet or normal chow, with or without EPO 50 μg/kg for 3 weeks (n=6) or 3 weeks plus 5 days of normal chow as recovery scheme (n=6); 6 mice were used for both control groups. At the indicated time points, mice were sacrificed. MOG gene expression was analysed by qPCR. No effect of EPO was observed neither at 3 weeks nor at 3 weeks plus 5 days of recovery with normal chow. MOG mRNA was expressed as arbitrary units *vs* control. Results are the average ± SD of samples analysed in duplicate. No effect of EPO on MOG induction was found at both the time points analysed.

7.2 Effect of EPO on myelin gene induction in the cuprizone model

Following the first pilot experiment described above, the study was repeated changing the time points analysed. The focus was now at 5 weeks of cuprizone treatment, as MOG gene expression was still lower than in the control group. The reason for choosing this time point was because, according to Morrel, it also represented the pick of demyelination that was not complete at 3 weeks (Morell et al., 1998).
Since in the previous experiment after 5 days of normal chow MOG increased again to control level, in this experiment two additional intermediate time points were chosen: 1 and 3 days of normal chow after 5 weeks of cuprizone insult.
The time point of 5 weeks was also chosen to allow the comparison and confirmation of results from the previous experiment.
The aim of this scheme was to study whether EPO inhibited demyelination (at 5 weeks) or improved the remyelination process (after 1 or 3 days of normal chow, when the remyelination process is started).
Consequently, the groups assessed for this experiment were:

6 mice CONTROL 3 mice CUPRIZONE 3 mice CUPRIZONE + EPO	**5 weeks cuprizone**
6 mice CUPRIZONE 6 mice CUPRIZONE + EPO	**5 weeks cuprizone + 1 day normal chow**
6 mice CUPRIZONE 6 mice CUPRIZONE + EPO	**5 weeks cuprizone + 3 days normal chow**

As shown in Fig. 7.3, the decrease of MOG gene expression by cuprizone insult at 5 weeks was confirmed (0.3 ± 0.04 CTRL *vs* 1 ± 0.1 cuprizone, P< 0.001).

However, as for the 3 weeks cuprizone diet, EPO did not have any effect on blocking demyelination.

In the group 5 weeks of cuprizone plus one day of recovery with normal food, MOG expression was still low and not significantly different from the 5 weeks cuprizone group (0.5 ± 0.1 at 5 weeks plus one day recovery *vs* 0.3 ± 0.04 at 5 weeks of cuprizone). There was no modulation of MOG by EPO at this time point of 5 weeks plus 1 day (0.5 ± 0.1 cuprizone *vs* 0.5 ± 0.04 cuprizone plus EPO).

At the final time point, 5 weeks of cuprizone plus 3 days of normal chow, the MOG expression returned to the control level (1.3 ± 0.1 cuprizone *vs* 1 ± 0.1 control). As in previous cases, EPO did not have any effect on induction of remyelination studied by MOG gene expression analysis (1.2 ± 0.1 EPO *vs* 1.3 ± 0.1 cuprizone group at 5 weeks plus 3 days) (Fig. 7.3).

In conclusion, the cuprizone model was set up properly but, unfortunately, EPO neither protected from demyelination nor stimulated remyelination as studied by myelin gene expression.

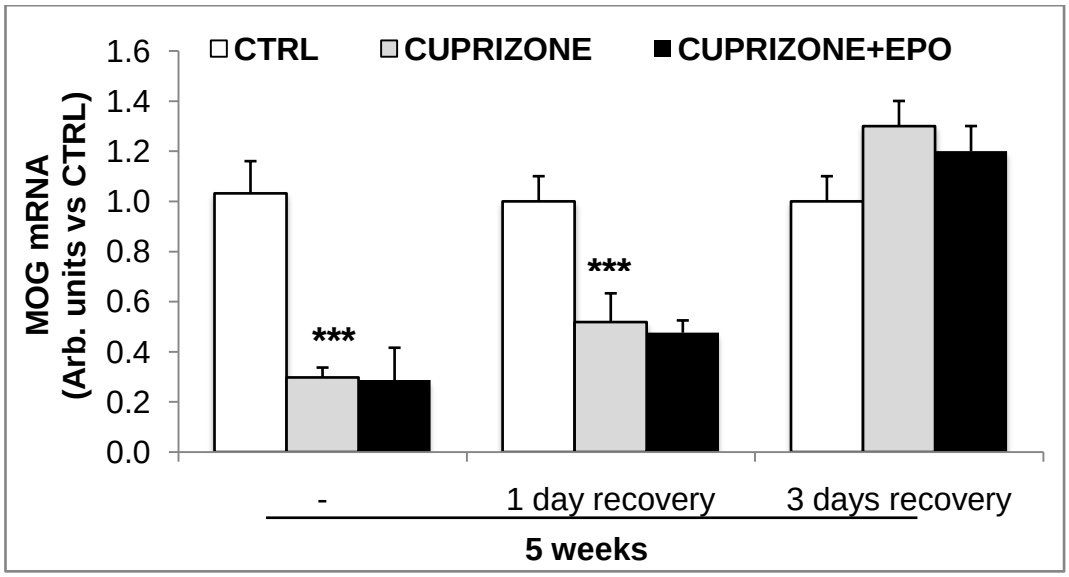


Figure 7.3: EPO did not have any effect on induction of remyelination in the cuprizone model. Mice were treated with or without 0.2% cuprizone diet and with or without EPO 50 μg/kg for 5 weeks (6 mice), 5 weeks plus 1 days of normal chow (12 mice) and 5 weeks plus 3 days of recovery (12 mice); 6 mice were used as control for all the groups considered. At the indicated time point, mice were sacrificed and the corpus callosum was isolated for mRNA extraction and qPCR for MOG gene expression analysis. Results are expressed as arbitrary units *vs* one control sample, and are average ± SD of n=6-12 mice. ***P<0.001 *vs* control. EPO did not have any effect on the remyelination.

7.3 Analysis of expression of inflammatory genes and of PDGFRα in the cuprizone model and effect of EPO

Since there was no effect of EPO on the modulation of MOG gene expression in the cuprizone model at any of the time points considered, analysis of other genes was undertaken to see whether EPO had an effect on some other pathways.

First of all, the expression of the platelet-derived growth factor receptor α (PDGFRα) was analysed. PDGFRα is a marker of oligodendrocyte precursor cells as NG2. The modulation of this gene was studied to investigate if EPO could induce the recruitment of precursor cells.

As shown in Fig. 7.4, there was no difference in PDGFRα expression between the control and the cuprizone group at 3 weeks (1.1 ± 0.1 control vs 0.9 ± 0.1 cuprizone), and EPO did not have any effect (0.9 ± 0.1 cuprizone + EPO vs 0.9 ± 0.1 cuprizone) (Fig. 7.4).

In addition, two inflammatory cytokines were analysed by qPCR. The first one was the chemokine (C-C motif) ligand 2 (CCL2) also known as monocyte chemotactic protein-1 (MCP-1). This cytokine is involved in the recruitment of monocytes, memory T-cells and dendritic cells to the sites of tissue injury, infection and inflammation. The time points analysed in this case were 3 and 5 weeks to see if there was any difference between the low peaks of the MOG expression and the expression of a cytokine involved in the neuroinflammatory process.

As shown in Fig. 7.5 A, at the time point of 3 weeks, there was a significant induction by cuprizone of CCL2 gene expression (3.2 ± 0.8 cuprizone *vs* 1.2 ± 0.2 control group). This increase was also observed at 5 weeks (5 ± 1.7 cuprizone *vs* 1.2 ± 0.2 ctrl group), and was not significantly different from the increase observed at 3 weeks. There was no effect of EPO at both the time points considered (Fig. 7.5 A).

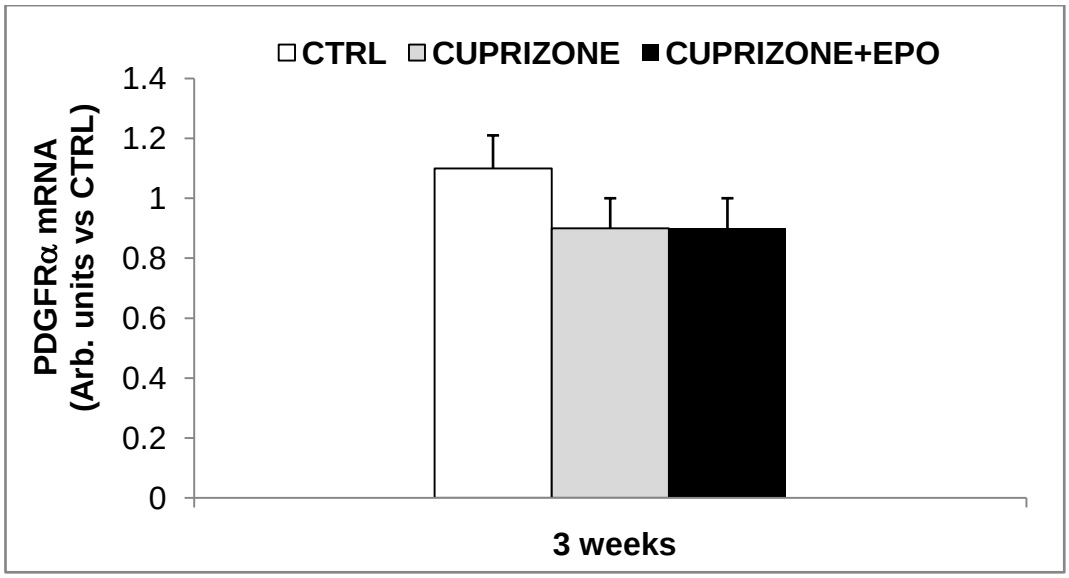


Figure 7.4: EPO does not modulate PDGFRα gene expression in the cuprizone model of demyelination. C57BL6 mice were treated with cuprizone with and without EPO 50 μg/kg. Mice were sacrificed after 3 weeks and the corpus callosum was collected as explained in legend to Fig. 5.3. qPCR analysis for PDGFRα was done in the samples from the 3 weeks time point (n=6). Results are expressed as arbitrary units *vs* one control ± SD.

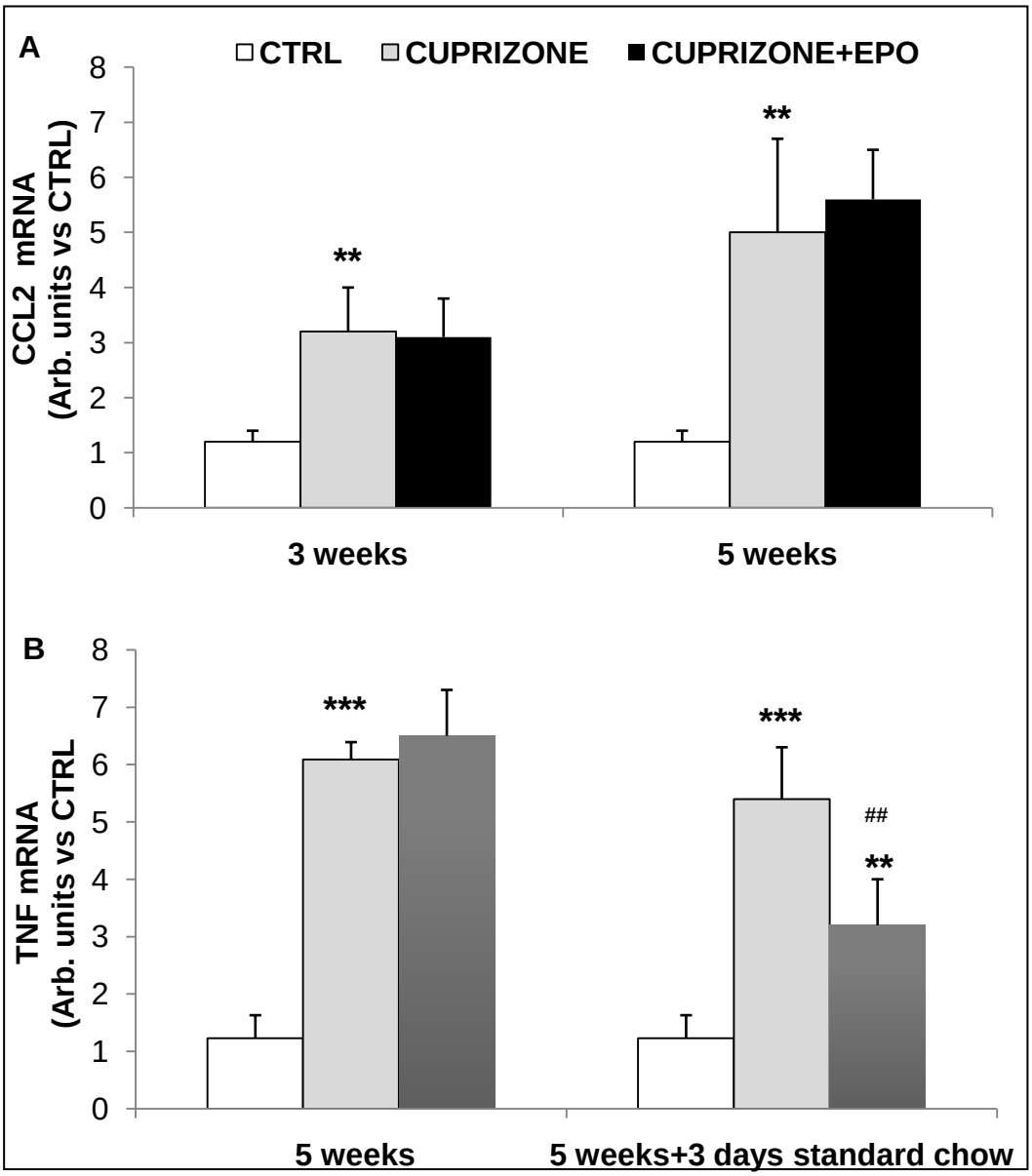


Figure 7.5: Effect of EPO on inflammatory cytokines. EPO slightly decreases only TNFα expression. Samples to be analysed were chosen from the previous two experiments as described in legend to Fig. 5.2 and Fig. 5.3 at the time point of 3 weeks and 5 weeks of cuprizone and 5 weeks plus 3 days of recovery. Results are expressed as arbitrary units vs one control ± SD. **P<0.01 and ***P<0.001 vs control; $^{\#\#}$P<0.01 vs cuprizone. EPO did not show any effect on modulation of CCL gene expression but slightly decreased the expression of TNFα after 3 days of recovery.

The last gene analysed was TNFα. It was studied at the time point of 5 weeks, at the peak of demyelination and at 5 weeks plus 3 days of normal chow, during remyelination, to study if EPO had an effect on the inflammatory pathway during repair.

As shown in Fig. 7.5 B, TNFα was dramatically induced at 5 weeks of cuprizone diet (6.09 ± 0.3 cuprizone vs 1.2 ± 0.4 control group) and still comparably high after 3 days of recovery (5.4 ± 0.3 cuprizone vs 1.2 ± 0.4 control group). No effect of EPO was observed at 5 weeks but a slight decrease of TNFα by EPO was found after 3 days of recovery with normal chow (5.4 ± 0.3 cuprizone vs 3.2 ± 0.8 EPO treated group). The effect of EPO was significantly different from both the control ($P< 0.01$) and the cuprizone group ($P<0.01$) (Fig. 7.5 B). This result obtained with EPO treatment confirmed the ant inflammatory role of EPO in the in vivo models of diseases. Maybe EPO was not able to inhibit the expression of TNFα during the cuprizone insult but it is able to improve the recovery in terms of inflammation when the cuprizone diet is stopped. In the 5 weeks cuprizone plus 3 days of normal chow group, the TNFα expression was still high even if the MOG gene expression was back to the control value.

The cuprizone study was stopped because it became clear that EPO had no effect detectable by qPCR gene expression analysis. In addition, at the same time this experiment was running, the Hagemeyer paper (Hagemeyer et al., 2012) was published looking at the effect of EPO on cuprizone by magnetic resonance imaging and behavioural tests, as it will be discussed in the last chapter.

7.4 Summary of chapter 7

This chapter was focus on translating *in vivo* the results previously obtained *in vitro* in CG4 cells. The aim of this part of the thesis was to demonstrate an effect of EPO on remyelination or in protection from demyelination.

An *in vivo* model of demyelination induced by cuprizone was set up in mice. In line with previous experiments in CG4 cells, MOG gene expression was analysed in demyelinated mice treated with or without EPO.

Several time points were considered. The first one was at three weeks of cuprizone treatment, since a low peak of myelin gene expression was reported at this time (Morell et al., 1998). The second one was at 5 weeks of cuprizone diet, since it corresponded to the maximum of demyelination (Morell et al., 1998). This two time points were focus on understanding whether EPO could counterattack the cuprizone induced demyelination. The model showed a decrease of MOG gene expression at both the time points but, unfortunately, EPO did not increase their expression.

Afterwards, the work focused on finding an effect of EPO on remyelination by increase of MOG gene expression during the recovery phase. In fact, when the cuprizone diet is stopped there is a spontaneous remyelination. Several time points were studied to this aim: 3 weeks of diet plus 5 days of normal chow and 5 weeks of diet plus 1 or 3 days of normal chow.

The 5 days of recovery was a too long time to study the effect of EPO, since the MOG expression was already back to the control values. At 1 and 3 days there was no effect of EPO on MOG induction compared to the group without EPO. In conclusion, no effect of EPO was found *in vivo* neither in protection from demyelination nor in induction of remyelination.

Finally, the effect of EPO on modulation of PDGFRα, a marker of precursor cells, and on some inflammatory genes was studied. EPO did not have any effect on modulation of PDGFRα.

As inflammatory cytokines, CCL2 and TNFα were analysed. EPO did not show any anti-inflammatory effect on CCL2. The only effect of EPO was a

slight reduction of TNFα in the group treated with cuprizone for 5 weeks and kept on normal chow for 5 days. At this time point there was a complete recovery of MOG gene expression but there was still a great induction of TNFα in the group without EPO treatment. This result confirms the anti-inflammatory effect of EPO *in vivo*.

Chapter 8: Discussion and conclusions

8.1 Effect of EPO on inflammation

The first topic studied in this thesis work was the anti-inflammatory effect of EPO *in vitro*, as part of its neuroprotective role.
It is well known that EPO decreases inflammation and cytokines production *in vivo* during diseases (Agnello et al., 2002a) (Villa et al., 2003). However, the anti-inflammatory effect of EPO *in vitro* is controversial.
According to Yazihan et al., EPO inhibits LPS-induced TNFα in the human monocytic cell line U937 (Yazihan et al., 2008). Nairz et al. similarly reported that EPO inhibits *in vitro* TNFα and IL6 production in LPS-stimulated primary mouse peritoneal macrophages (Nairz et al., 2011).
On the other hand, EPO did not have any direct anti-inflammatory effect on TNFα induced by injection of LPS in mice neither ip (Mitra et al., 2007) nor icv (Villa et al., 2003). In addition, a study in healthy volunteers injected with LPS, with and without EPO, reported that EPO increased TNFα and IL6 levels in plasma (Hojman et al., 2009). *In vitro*, EPO did not decrease LPS induced TNFα in microglia and PBMCs (Wilms et al., 2009) (Villa et al., 2003).
However, according to Villa et al., EPO decreased apoptosis-induced TNFα in neuronal cells instead (Villa et al., 2003), showing that the anti-inflammatory effect of EPO could rather be a consequence of the anti-apoptotic one.
The effect of EPO on inflammation was further investigated in this work, to find whether EPO can act as a direct anti-inflammatory molecule, maybe through some other pathways differently acting compared to bacterial agents.
EPO did not show any anti-inflammatory effect in term of modulation of inflammatory cytokines induced by danger signal molecules neither in a

monocytic cell line (MM6) nor in primary cells (PBMCs), at all doses investigated.
A scheme of danger signals used is showed in the table below:

Danger signals	Genes analysed	Cells used
LPS	IL6	Hu PBMCs
HMGB1	IL6	MM6
HMGB1	IL6	Hu PBMCs
NCL±LPS	IL6	MM6
ATP±LPS	IL-1β	MM6

HMGB1 was reported to stimulate proinflammatory cytokine synthesis in human monocytes (Andersson et al., 2000). The necrotic cell lysate increased TNFα and IL6 expression when associated with LPS (El Mezayen et al., 2007). ATP, in association with a priming stimulus, like LPS or TNFα, triggered the secretion of mature IL-1β by activation of the inflammasome (Franchi et al., 2009).
The lack of the EPO effect in these cells was not due to a lack of EPOR expression. In fact, it was already reported that PBMC cells express EPOR (Lisowska et al., 2010) and we found that MM6 cells express EPOR to levels comparable to those of PBMCs.
Afterwards, we demonstrated that EPO did not act as anti-inflammatory by inhibiting the expression of the pro-inflammatory receptor TREM-1. The effect of EPO on this receptor was investigated since, as reported by Mengozzi et al., its expression was decreased by EPO in an *in vivo* model of stroke in which EPO was protective (Mengozzi et al., 2012).
We also excluded that an anti-inflammatory effect of EPO could arise from the mobilization of endothelial precursor cells from the bone marrow into the circulation. In fact, EPO pre-treatment in mice for 3 days was able to induce mobilization of precursor cells (Heeschen et al., 2003), that mediate angiogenesis but could also have an anti-inflammatory function.
Finally, we studied the effect of EPO on cytokines secreted by human macrophages upon stimulation with LPS (acting through TLR4) and other stimuli acting on different TLR (R848 binding TLR7 and TLR8; PAM3

binding TLR2/1) or partially stimulating the TLR pathway (IL-1α and IL-1β). EPO did not decrease the expression of cytokines induced by these pathways and did not show any anti-inflammatory activity also in this model.

Two main reasons will try to justify why, in this study, an *in vitro* anti-inflammatory effect of EPO was not found.

The first explanation would be, according to Villa, that the anti-inflammatory role of EPO observed *in vivo* is secondary to the anti-apoptotic one (Villa et al., 2003).

The second explanation is related to the problem of the receptor mediating EPO effect on neuroprotection that is still controversial.

EPOR is required in neuroprotection (Yu et al., 2001) (Um et al., 2007), its expression is increased in diseases (Cho et al., 2012) (Siren et al., 2001b) (Bernaudin et al., 1999) and its up-regulation also mediated the protective phenomenon of the hypoxic preconditioning (Chen et al., 2010). Hovewer, Xiong reported that EPO provides neuroprotection also in the absence of EPOR in neuronal cells (Xiong et al., 2010) and Brines reported that derivatives of EPO that do not bind EPOR are still neuroprotective (Brines et al., 2008) (Leist et al., 2004). Therefore, another receptor might be involved in mediation of neuroprotection by EPO. The main hypothesis is the dimeric receptor formed by βcR and EPOR, since an effect of EPO through this receptor has been demonstrated. According to Brines and Cerami, it would be more accurate to speak about a tissue-protective receptor (TPR), mainly composed of βcR and EPOR, that is not expressed in normal tissues but is activated after injury (Brines and Cerami, 2012).

In conclusion, the second reason why the anti-inflammatory effect of EPO was not observed *in vitro* could be the lack of the receptor in physiological conditions. Maybe this EPO effect can be only observed during diseases, when the proper EPO-receptor binding (with the homodimeric EPOR or the TPR) can happen.

8.2 EPO on myelin gene induction

The second topic studied in this work was the effect of EPO on induction of myelin gene expression.

As discussed in the introduction, EPO has a protective role *in vivo* in the multiple sclerosis animal model, by decreasing the severity of the disease and the expression of inflammatory cytokines (Savino et al., 2006) and reducing the inflammatory cell infiltration (Agnello et al., 2002a). However, EPO also increases oligodendrocytes proliferation (Zhang et al., 2010), promotes oligodendrogenesis and attenuates white matter injury in rats following hypoxia/ischemia. *In vitro*, Sugawa et al. showed that EPO increases the number of MBP-positive cells in oligodendrocytes culture (Sugawa et al., 2002).

Therefore, the aim of this investigation was answer the question: is EPO only acting as an anti-inflammatory cytokine or is also involved in repair by inducing remyelination?

We found an effect of EPO on induction of myelin gene expression (MBP and MOG) in differentiating CG4EPOR cells, a line of oligodendrocyte precursors modified to overexpress EPOR. The expression of MOG protein was also induced by EPO. However, EPO did not have any effect on induction of these genes in CG4 WT cells that did not express EPOR.

CG4 cells were used as tool to study EPO on myelination because they are considered a model of myelination *in vitro* (Annenkov et al., 2011) (Wang et al., 2011) and also because CG4EPOR cells were available to study the involvement of EPOR in the mediation of this effect. Although CG4EPOR cells were a modified cell line, several reasons supported their choice as model of myelination instead of CG4 cells: oligodendrocyte cells express EPOR (Sugawa et al., 2002), EPOR expression is increased in pathological conditions (Taoufik et al., 2008) and also during demyelination (Cho et al., 2012) and we demonstrated that CG4EPOR cells had the same behaviour as CG4 WT cells in term of myelin gene expression during differentiation.

This effect on induction of myelin genes is important because it could be correlated to an induction of myelination. In fact, MOG and MBP are two

major components of myelin and are induced only by mature oligodendrocytes mainly after the onset of myelination. EPO effect on myelin genes can be detected at day 2 of differentiation, that is the minimum time CG4 cells need to start to acquire a phenotype typical of oligodendrocytes (Louis et al., 1992). In addition, we found that CG4EPOR cells were responsive to another myelinating agent (LIF), known to induce myelination *in vitro* (Ishibashi et al., 2006) and *in vivo* (Marriott et al., 2008). This result supported the hypothesis that the effect of EPO on induction of myelin genes could be translated into a myelinating effect. EPO had an effect on myelin gene induction in CG4EPOR cells but not in CG4 WT cells; therefore EPOR was necessary to mediate this effect. Moreover, this effect was not a consequence of the cell manipulation since there was no effect of EPO in CG4 cells transfected with an empty vector. To study the role of EPOR in this effect, we tried to induce EPOR in CG4 WT cells, by hypoxia or cloning cells by limiting dilution to isolate clones with higher expression of EPOR. We did not find any effect of EPO in all these experiments. However, when we cloned CG4EPOR cells by limiting dilution, we demonstrated a correlation between the expression of EPOR and the effect of EPO on myelin gene induction. In fact, in CG4EPOR clone R15, expressing a higher amount of EPOR than clone R9, the induction of MOG by EPO was greater than in clone R9.

The comparison of EPOR expression in the different clones of CG4 WT and CG4EPOR cells was really interesting. Clone WT3 was the CG4 WT cell clone expressing the highest amount of EPOR that we could use among all the isolated clones, but it was still not responsive to EPO. We then realized that EPOR expression in WT3 clone was about 1000 fold lower than in CG4EPOR cells; more interestingly, it was about 10 fold lower than EPOR expression in the CG4EPOR clone R9, representing one of the CG4EPOR clones with the lowest expression of EPOR, however responsive to EPO. This observation may explain the non responsiveness of CG4 WT cells, and of CG4 WT cell clones, to EPO, but it also suggest that the expression of EPOR required by EPO to induce myelin genes does not need to be massive like in the CG4EPOR cell line.

A further investigation was also done in primary oligodendrocytes to confirm the results obtained in the cell line. EPO did not have any effect on induction of myelin genes in these cells.
The expression of EPO receptor was analysed by qPCR in primary oligodendrocytes precursors (OPC) and was compared with the expression of the receptor in the CG4 cell lines. Primary OPCs expressed about half the amount of EPOR compared to clone R9, the CG4EPOR clone with the lowest expression of EPOR but responsive to EPO. Therefore, the reason why primary oligodendrocytes are not responsive to EPO can be explained with a too low expression of EPOR in these cells in physiological conditions.
However, as reported before, there is an induction of EPOR in pathological conditions and when quantified this was about 3.5 folds (Cho et al., 2012). In conclusion, there are reasons to believe that EPOR expression in pathological conditions can be high enough to have an effect by EPO on myelin gene induction.
The study of the involvement of EPOR in mediation of EPO effect on myelin gene induction is important to solve the open issue of which receptor mediates EPO neuroprotection. In fact, although EPOR mediates EPO effect in erythropoiesis, derivatives of EPO that do not bind EPOR are still neuroprotective. Therefore, another receptor should be involved in mediation of this effect (Brines et al., 2008). Recently, Sinclair et al. also debated whether EPO and EPOR could have an effect in neuroprotection (Sinclair et al., 2010). With this study, we add another piece of information about the importance of EPOR in mediation of EPO effect in myelination. The results obtained here are in line with those obtained by Cho et al., who found that EPO promotes oligodendrogenesis and myelin repair following lysolecitin-induced injury in spinal cord slice cultures. Of note, they also found that the use of a neutralising anti-EPOR antibody suppressed the beneficial effect of EPO in remyelination (Cho et al., 2012).

As part of the study of EPO on myelin gene induction, intracellular pathways activated by EPO leading to this effect were also investigated.

The role of the early growth response gene EGR2 was analysed at first for two reasons: because EGR2 is required for the peripheral nervous system myelination (Topilko et al., 1994) and because in a model of stroke, in which EPO was protective, EGR2 was one of the main genes induced by EPO (Mengozzi et al., 2012). Following stroke, there is also death of oligodendrocyte cells and a white matter injury. Therefore, an effect of induction of EGR2 by EPO in this model could be connected with a role in myelination. Actually, the only data about a possible role of EGR2 in the CNS myelination was that EGR2 KO mice did not show any defects in CNS myelination (Topilko et al., 1994) and EGR2 was not detected in oligodendrocyte cells (Sock et al., 1997). However, these were the only two studies published and the detection of EGR2 in oligodendrocytes was done by Northern blot that is not as sensitive as qPCR. Therefore, we checked whether EGR2 was expressed in CG4EPOR cells. It was expressed at a basal level and also induced by 1 hour of EPO treatment. The hypothesis was that EPO could induce myelin genes through induction of EGR2. However, by silencing EGR2 gene expression, EPO still induced MBP gene expression after 4 days of differentiation, showing that this gene is not involved in the pathway that mediates the induction of myelin genes by EPO.

Although EGR2 did not mediate EPO-induced myelin gene induction, the interesting result was that the silencing of EGR2 gene expression increased MBP expression. This result was not a consequence of the siRNA used to knock down the gene since using a negative control there was no induction of MBP. This is a really interesting result that should be further investigated, since EGR2 could be involved in myelination in the CNS with an opposite effect compared to that in the PNS.

Moreover, EGR2 was never found expressed in oligodendrocyte cells in the CNS; therefore it would be important to understand its role in these cells.

However, since EPO still induces myelin gene expression when EGR2 is silenced, the conclusion is that EPO might activate two different pathways in oligodendrocyte cells: the first one is the induction of EGR2, maybe

leading to some other effect rather than induction of myelination; the second one is another pathway leading to induction of myelin genes.
The basal expression of EGR2 was also confirmed in primary oligodendrocytes, although EPO did not have any effect on its induction, maybe for the same reason explained above (a too low expression of EPO receptor in primary oligodendrocytes in physiological conditions to see an effect by EPO).
The second pathway investigated was mTOR, a downstream signal of PI3K/Akt pathway. This pathway was involved in oligodendrocytes differentiation and also mediated some EPO effects. However, the rapamicin analogue KU 0063794, used to inhibit mTOR, could not be studied as planned since it was toxic for CG4 cells. Further study of this pathway, by silencing mTOR with an siRNA, could be done to investigate its involvement in mediation of myelin gene induction by EPO.

Finally, in an *in vivo* model of demyelination induced by cuprizone, EPO did not show any effect on myelin genes expression neither in protection from demyelination nor in induction of remyelination. The first effect was investigated at a time point of 3 weeks in which a low peak of myelin gene expression was demonstrated (Morell et al., 1998) and at 5 weeks that was the peak of demyelination (Morell et al., 1998). The second one was investigated after the interruption of the cuprizone diet, when there was a spontaneous recovery.
However, while these investigations were done, a study about the effect of EPO in a cuprizone model was published (Hagemeyer et al., 2012). This paper showed an effect of EPO in the improvement of sensitive motor coordination and vestibulomotor functions, apart from an anti-inflammatory effect of EPO in microglial cells. These effects of EPO were found with more sensitive analysis (MRI) compared to the study of gene expression by qPCR. For this reason we interrupted our studies without finding the best time point to study EPO effect on myelin genes. We believe that EPO can have an effect on remyelination but not so strong to be detected *in vivo* by qPCR in brain regions.

In conclusion, EPO showed an effect on induction of myelin gene expression in the oligodendrocyte cell line and this effect was mediated by EPOR. In primary oligodendrocytes this effect was not confirmed but the expression of EPOR was not high enough to expect an effect of EPO. The translation of these results into the *in vivo* model of demyelination was interrupted only because an effect of EPO in this model was demonstrated by deeper analysis (Hagemeyer et al., 2012).
The main questions that are still open are about the role of EGR2 and mTOR in CNS myelination. Since EGR2 is expressed in oligodendrocyte cells and since EGR2 knockdown increases myelin gene expression, it could be also involved in CNS myelination.

As future work, it would be of interest to investigate:

- The role of EGR2 in myelination.
  Although EGR2 is not involved in mediation of EPO effect on myelin gene induction, it can be involved in the central nervous system myelination. In fact, the increase of myelin gene expression by silencing EGR2 showed that it can play an opposite role compared to the one in the peripheral nervous system, where EGR2 expression is required for myelination. This study could be done in primary oligodendrocyte cells or in *in vivo* models of demyelination/remyelination. The EGR2 pathway could be investigated by blocking EGR2 with an siRNA, as done in CG4EPOR cells.
- The role of EPO in myelination in primary oligodendrocytes.
  It would be of interest to confirm the effect of EPO on myelin gene induction also in primary oligodendrocytes. However, since in physiolological conditions EPOR is expressed at a very low level in primary cells it would be necessary to use a model of demyelination to increase EPOR expression and to confirm the effect of EPO.
- The role of mTOR in mediation of myelin gene induction by EPO.
  To this aim an mTOR siRNA could be used to block mTOR gene expression in CG4EPOR cells.

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