# UPSTREAM PATHWAYS REGULATING ERYTHROPOIETIN GENE EXPRESSION IN THE LIVER DURING ACUTE PHASE RESPONSE: A CENTRAL ROLE FOR IL-6

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***To Luigi, tireless worker and precious confidant.***

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**SUMMARY.**

Erythropoietin (EPO) is a 30-35kDa glycoprotein hormone that has been long appreciated for its effect of maintaining the circulating erythrocytes mass. In recent years EPO has been recognized as a member of the cytokine type 1 superfamily. In fact, EPO has multiple functions outside the bone marrow, many of which parallel its action in haematopoiesis, where it functions to promote pro-erythroblast survival and maturation. In mammals, during the foetal life, the liver represents the major producer of erythropoietin in the whole organism and the main centre of erythropoiesis. After birth the kidney is supposed to become the most important Epo-producing organ through a molecular switch that is not yet fully understood; however, in times of hypoxic stress the liver still contributes significantly. Since EPO has been shown to be induced during hypoxia, its transcriptional regulation has been described to be dependent mainly on oxygen-dependent regulatory mechanisms. Erythropoietin gene expression is induced by hypoxia via the hypoxia inducible factor (HIF) family of transcription factors (mainly HIF-1α and HIF-2α). Most of the studies concerning erythropoietin gene regulation have been performed under hypoxic conditions whereas the Epo-gene expression and its transcriptional regulation have not been clearly investigated during acute phase response.

The acute phase response is a complex reaction to various stressful stimuli such as surgery, wounding, bacterial or virus infection, or elevated levels of stressful and tissue-damaging agents. During this mammalian stress response, the plasma levels of a group of proteins change rapidly. These proteins are called the acute phase proteins (APPs). The centre of APPs production is the liver which synthesizes and secretes most of them into the bloodstream. Although the precise functions of many APPs are still partially unknown, in general they shared a common function that consists in maintaining the balance of organism homeostasis under stressful conditions, other than eliminating infectious agents and limiting the damage induced by the inflammatory reaction. The changes involved in the organism response to homeostatic disturbances comprehend a wide range of metabolic alterations interesting the whole body, like changes in lipid and iron metabolism,

hypoferremia/ hypozincemia, increased gluconeogenesis, increased muscle protein catabolism and transfer of aminoacids from muscle to liver, activation of the complement and coagulation pathways and hormonal changes. The main mediators responsible for all these metabolic alterations are the acute phase cytokines (mainly IL-6, IL-1β and TNF-α), which are produced in the site of injury and reach several body districts through bloodstream. The clinical parameters which represent this condition are leukocytosis, thrombocytosis and erythrocytosis, often accompanied by fever, somnolence and weakness.

The analyses performed in the present work emerged from the observation that during several clinical conditions characterized by acute phase reaction the circulating levels of erythropoietin augment. The detection of an increase of the EPO concentration in the serum of rats submitted to an experimental model of acute phase, intramuscular injection of turpentine oil (TO), prompted to identify the possible sources of this hormone under these specific normoxic conditions. Once analyzed the main potential producers, the liver emerged as the major contribute to the total circulating erythropoietin in terms of mRNA expression. In parallel to erythropoietin expression, an increase of the gene expression and of the protein levels of the main transcriptional regulators HIF-1α and HIF-2α was detected in the liver of rats submitted to muscular injury accompanied by an augment of their DNA binding activity. Challenging primary cultures of rat hepatocytes with acute phase cytokines revealed an up-regulation of erythropoietin gene expression particularly evident following IL-6 treatment, whereas TNF-α treatment induced a dose-dependent down-regulation of the gene. A similar response was also reported for Kupffer cells, resident macrophages of the liver, and a human hepatoma cell line, Hep3B. In the first part of the study EPO turned out as a positive acute phase protein and a possible role of IL-6 in the erythropoietin gene regulation during acute phase reaction was suggested.

In order to clarify the role of IL-6 in this context a second serie of experiments was performed with the use of IL6 knockout mice and the respective wild type strain. Moreover the study was enriched with the introduction of a further experimental model of acute phase, intraperitoneal

lipopolysaccharide (LPS) administration, in order to compare two different patterns of mediators characterizing the acute phase response. Interestingly, the deficiency of IL-6 strongly reduced erythropoietin gene up-regulation observed in the liver of wild type animals during both experimental conditions and the erythropoietin serum concentration in IL6KO mice was significantly lower compared to the wild type strain. Furthermore, whereas the hepatic gene and protein levels of HIF-1α resulted also reduced in the knockout strain, particularly in TO treated animals, HIF-2α expression was increased during acute phase in both mice strain regardless of the lack of IL6. Indeed, the bandshift analysis revealed an increase of HIFs DNA binding activity in the knockout strain too during acute phase reaction. The localization of both transcription factors in the liver through immunofluorescence revealed an increase of nuclear signal for HIF-1α dominantly expressed in hepatocytes, while HIF-2α was mainly identified in non-parenchymal cells of the liver, reasonably macrophages, endothelial cells or fibroblasts. These data were supported with an in vitro analysis of the effects of IL-6 in erythropoietin synthesis in mouse hepatocytes and in the HIFs member gene and protein expression.

The results emerging from the present study indicate the liver as the major source of erythropoietin during acute phase conditions and IL-6 turned out as the principal mediator capable to induce the expression of the hormone in hepatocytes, offering a new insight in the regulatory mechanisms of Epo gene regulation. The comparison of two different models of acute phase indicated that erythropoietin gene expression might be regulated irrespective of HIF-1α and HIF-2α activity that resulted to be induced during acute phase reaction but localized in different cell populations of the liver. The involvement of HIF-1α in the regulation of several genes activated in both acute phase response and hypoxia might suggest a key role of this transcription factor in cellular adaptation to stressful conditions.

# 1 INTRODUCTION.

## 1.1 A HUNDRED YEARS OF ERYTHROPOIETIN RESEARCH.

### *1.1.1 Brief Historical Introduction*

The concept of an hormonal regulation of erythropoiesis was first hypothesized by Paul Carnot, Professor of Medicine at the Sorbonne, Paris, and his co-worker Deflandre in 1906 (1). They developed experiments on rabbits subjected to bleeding (about 30 ml) taking another blood sample from these animals one day later and injecting the serum (5-9ml) into normal rabbits. The concentration of red blood cells in the recipients increased up to 40% within a couple of days. They concluded in this way that the serum contained a haematopoietic factor that they initially called hemopoietine. Several years later, in 1936, Erling Hjort (2) confirmed the observations of Carnot and Deflandre's work enlarging the investigations to a total number of 18 experiments and 5 control animals. Further studies have then shown that reticulocytosis occurs after 3-4 days from erythropoietin serum levels increase and the concentration of red blood cells requires even longer period to rise significantly. The more specific name "erythropoietin" for this hormonal substance was introduced by two scientists, Eva Bonsdorff and Eeva Jalavisto, in 1948 (3). However, the most important contribute to the identification of Epo is attributed to the studies of Allan Erslev, a Professor of Medicine at Thomas Jefferson University in Philadelphia, who in 1953 transfused large volumes of plasma from anaemic rabbits into normal rabbits, observing a significant increase of reticulocytosis and an increase in hematocrit values. "Conceivably isolation and purification of this factor would provide an agent useful in the treatment of conditions associated with erythropoietic depression, such as chronic infection and chronic renal disease" (4). Allan Erslev has been considered one of the pioneers in erythropoietin research.

### *1.1.2 Chasing a ghost: isolation and characterisation of EPO.*

The existence of erythropoietin was not recognized for several years and the results obtained by Carnot and Deflandre were extremely difficult to reproduce in the described way. The effective slow progress in Epo investigation is comprehensible in view of the low concentration of the hormone in biological fluids and tissues, which rendered its detection difficult (5). In fact, under physiologic condition 1L of human plasma contains about 50ng of Epo, and based on their experience with the extraction of the hormone from sheep plasma, Goldwasser and Kung (6) measured that a volume of 3250 L of urine from anaemic patients was required to purify 10mg of pure human Epo, which they calculated it would have represented a 3 years daily urine collection from a single patient, or one month's collection from 36 patients.

The pure human urinary Epo prompted to the identification of its amino-acid sequence and the subsequent characterization of the human gene (7). The EPO gene is located on chromosome 7, exists as a single copy in a 5.4-kb region of the genomic DNA, and encodes a polypeptide chain containing 193 amino acids. During the synthesis and secretion of EPO, a 166 –amino-acid peptide is initially generated following a cleavage of a 27 amino-acid hydrophobic secretory precursor at the amino-terminal. Moreover, a carboxy-terminal arginine in position 166 is removed both in the mature human and recombinant human EPO, resulting in a circulatory mature peptide of 165 amino acids (8). Once cleavages are performed, EPO becomes a 30.4-kDa glycoprotein with approximately 50% of its molecular weight derived from carbohydrates that can differ among species. The N-glycans (fig.1) are critical for the *in vivo* biological activity of Epo, and particularly important are the terminal sialic acid residues of these glycans (9). Like other asialo-glycoproteins, asialo-Epo is rapidly catabolized via galactose receptors present on the hepatocyte membranes, because galactose is the preterminal sugar of the glycans.

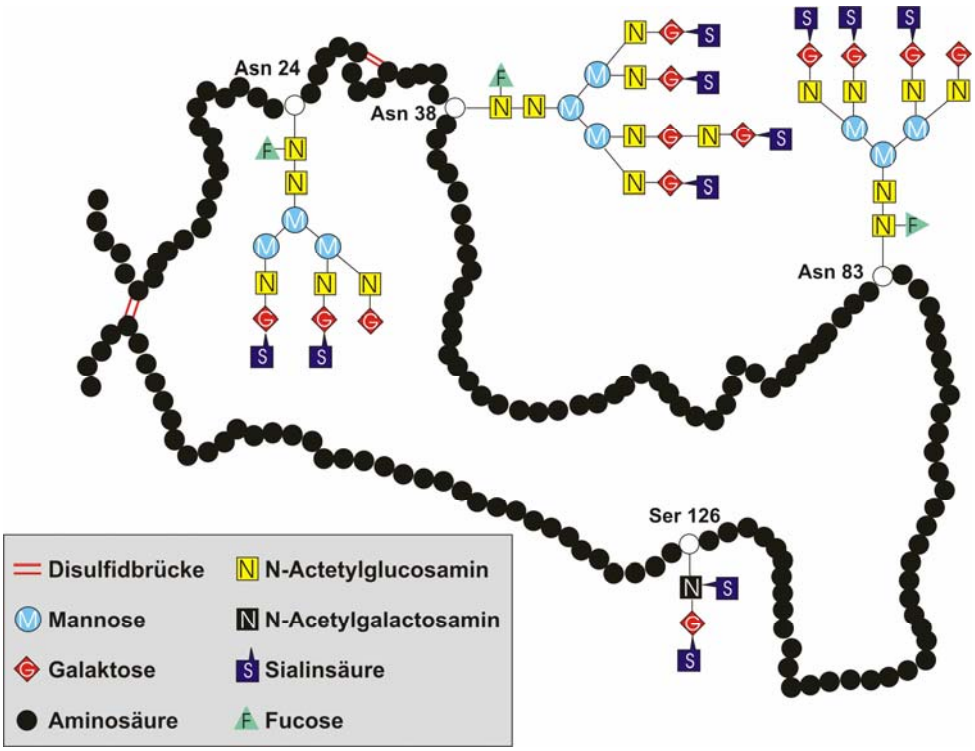



***Figure 1. Human Erythropoietin glycosylation sites (Marc Machnik, Bettina Bialas und Wilhelm Schänzer, Institut für Biochemie, DSHS Köln).*** *The aminoacids residues in position Asn24, Asn38, Asn83 and Ser126 are important for the biological activity of the glycoprotein. These glycosylated residues interact directly with the receptor.*

### *1.1.3 Stimulation of erythropoiesis by hypoxia: kidney but more.*

One of the most important papers proving the existence of Epo was based on the works by Kurt Reissmann (10) who in 1950 demonstrated in parabiotic rats that when one partner is exposed to hypoxia whereas the other partner exposed to an atmosphere at normal oxygen tension, both animals developed erythroid hyperplasia in their bone marrow. From these data the primary function of Epo appeared so to maintain the blood haemoglobin concentration in the normal range during steady-state conditions and to stimulate red cell mass recovery after haemorrhagic conditions. In fact, the concentration of circulating Epo augments exponentially with decreasing

haemoglobin levels during anaemia. The kidney results very appropriate for controlling Epo production because the oxygen tension in the renal cortex is minimally affected by the rate of blood flow as the renal $O_2$ consumption changes in proportion with the glomerular filtration rate (11). The important role of the kidney in the production of Epo was suggested by Leon Jacobson and his colleagues who in 1957 published that nephrectomised rats show defects in responding to the normal increase in the plasma Epo level on hypoxic stress (12). Jacobson's group aimed at the elucidation of the site of Epo production, having removed the pituitary gland, spleen, 90% of the liver, adrenals and the gonads before finding that only bilateral nephrectomy failed to respond to cobalt and bleeding in increasing Epo concentrations in plasma. On the basis of these invetigations, Fisher and Birdwell (13) successively detected erythropoietic activity on *in situ* perfused dog kidneys. However, for several years it was not clear whether renal cells produce erythropoietin because other attempts failed to extract Epo from the kidney. Successive studies using cDNA probes with in situ hybridization techniques to detect mRNA for Epo revealed high levels of mRNA in the interstitial cells of the mouse kidney following an haemorrhagic phenomenon (14). With the development of more sensitive bioassays for erythropoietin, it became apparent that anephric animals, including humans, were anyway able to produce small but detectable amounts of erythropoietin in response to hypoxia (15-16). Furthermore, anephric foetal animals have been observed to produce almost the same amount of erythropoietin as control ones, and the extra-renal sites (mainly the liver) have been demonstrated to contribute to the synthesis of the hormone (17). In addition to the kidneys, the adult liver has also shown to produce erythropoietin under hypoxic conditions, with a potential contribution up to 80% after nephrectomy in rats (18). An interesting and not yet resolved difference between renal and hepatic EPO regulation is the fact that hepatocytes seem to be capable of augmenting the hormone expression at a cellular level, whereas in the kidney, up-regulation depends mainly by increased recruitment of a larger number of cells with a fixed amount of Epo mRNA (19). Further studies included in the extra-renal sources of Epo

during hypoxia also spleen, lung, heart, lung and bone marrow that showed low levels of Epo mRNA (20).

### *1.1.4 EPO-EPOR: pathways downstream the receptor.*

Erythrocytic progenitors in the bone marrow constitute the principal targets of EPO. The physiologically low concentrations of the glycoprotein enable only a small percentage of progenitors to survive whereas the remaining progenitors undergo apoptosis. For this reason, the primary mechanism through which Epo influences erythropoiesis is the prevention of programmed cell death (21). When Epo concentration augments in blood many more burst-forming unit-erythroid (BFU-E) and several colony-forming units-erythroid (CFU-E) escape from apoptosis and proliferate with a consequent induction of the growth and maturation of proerythroblasts and normoblasts. The mature EPO receptor is a 484 aminoacid glycoprotein which is a member of the cytokine class I receptor superfamily (22). Two of the membrane Epo receptor molecules organize to form a dimer to which one Epo molecule binds. Ligand binding induces a conformational change and a tighter connection of the two receptor molecules. As a result, two Janus kinase 2 (JAK2) tyrosine kinase molecules, which are in contact with the cytoplasmic region of the Epo receptor are activated (23). In chain, several tyrosine residues of the EPO receptor are phosphorylated exposing docking sites for signalling proteins containing SRC homology domains (SH2). Consequently, several signal transduction pathways are triggered, including phosphatidyl-inositol 3-kinase (PI-3k/Akt), JAK2, STAT5, MAP kinase and protein kinase C (24-25).

Inhibition of apoptosis appears to be a critical mechanism not only for the erythroid cell lines survival but also for the tissue-protective effects that erythropoietin has been recently revealed to explicate (26).

### *1.1.5 Extra-hematopoietic effects of EPO.*

In addition to its principal role in the regulation of mammalian erythropoiesis, Epo signalling has emerged as a major tissue-protective survival pathway in various non-haematopoietic organs.

Alterations of Epo-EpoR signalling in mice leads to in utero death between embryonic days E11.5 and E13.5 because of a lack of definitive erythropoiesis in the foetal liver and severe anaemia (27). The identification of Epo receptor expression in non-hematopoietic tissues such as the brain and heart has suggested a role for Epo signalling in the embryonic development of specific non-haematopoietic organs. The discovery of increased apoptosis in the myocardium and in the brain of Epo receptor-null mouse embryos indicated an important role for Epo signalling in tissues development (28). The neuroprotective effect of exogenous Epo as well as the presence of hypoxia-inducible Epo expression in the adult rat brain, the primate brain and in primary cultured astrocytes (29) suggested that endogenous brain Epo-EpoR signalling may be important for neuronal survival. EpoR expression in various types of vascular endothelial cells has been associated with the ability of Epo to promote migration and proliferation of endothelial cells in different *in vitro* experimental models (30). A series of recent studies has proved that Epo administration exerts significant cardioprotective effects during ischemic injury in various pre-clinical experimental models including ischemia/reperfusion injury in isolated, ex vivo perfused hearts and in permanent or transient coronary artery ligation models in vivo (31). Several studies have investigated the ability of Epo to affect immunological responses, but the mechanisms of the immunological and anti-inflammatory effects of Epo still require further characterizations.

## 1.2 IDENTIFICATION OF THE EPO-PRODUCING ORGANS

### *1.2.1 Definitive erythropoiesis in the embryonic liver: the switch after birth.*

In human, primitive erythroblasts enter the embryo during the fourth week of gestation as the yolk sac vasculature connects with the dorsal aorta and the heart begins to contract. Soon afterwards,

hematopoietic cells begin to invade the newly forming liver (32). The liver serves as the major site of haematopoiesis throughout the first trimester of the gestational period. Erythroid cells in close association with macrophages differentiate in the liver, loosing their nuclei prior reaching the bloodstream. During foetal life erythropoietin mRNA has been detected in the liver and erythropoietin prevents apoptosis in foetal erythroblasts. Animal studies on nephrectomised or hepatectomized foetuses or adult animals of different mammalian species (rat, mouse, and sheep) showed clear differences in the patterns and the dynamics of the switch from the hepatic Epo production to the renal one after birth in relation to gestational age and maturity. Whereas specific differences in timing of the switch and the grade of contribution to the total body Epo mRNA content exist (33), the switch has been observed in human as well. The mechanisms and the main factors determining the switch still remain to be identified, but recent data indicate that GATA-4 may be one of the factors that specifically promote Epo tissue expression in the liver (34). It is reasonable to believe that tissue factors in the adult kidney may well rise around birth that are indispensible for Epo expression and repressive factors are expressed in the liver of the adult. The specific identification of renal EPO-producing cells and a setup of cell-specific Epo regulation in the liver have not been enough investigated.

### *1.2.2 Extra-renal sources of erythropoietin.*

After the studies of Fisher and co-workers, the cloning of EPO cDNA provided new approaches to measure EPO mRNA. Northern blot analysis showed that hypoxia or cobalt-induced increases of serum Epo correlate with those of renal Epo mRNA, which indicates that Epo production is regulated by its mRNA level, and not stored in the cell (35). By *in situ* hybridization technique, mouse Epo mRNA was detected mainly in the inner cortex of the kidney and the cells accumulating Epo mRNA under hypoxia appeared to be interstitial cells in the peritubular capillary bed. Epo mRNA in these interstitial cells has been shown to co-localize with ecto-5'-nucleotidase,

identifying them as fibroblast/like cells (36). With the same technique, hepatocytes located around the central veins of the liver have been shown to accumulate transgene-induced Epo mRNA. Interesting observations moved by Fried (37) in an analysis of erythropoietin production by extra-renal sites suggested the hypothesis that this phenomenon could be regulated primarily by the rate oxygen supply/oxygen demand as is that interests the kidney, but extra-renal sites produce only a fraction of the amount of erythropoietin produced by the kidneys in response to comparable conditions. Indeed, he showed two experimental conditions resulting in profound increments in the plasma erythropoietin levels of hypoxic anephric rats: interventions that cause liver damage and the administration of rennin or angiotensin. The observations reported in the mentioned study were also repeated by other groups who proved that liver injury by partial hepatectomy, CCl4 administration and bile duct ligation in rats were able to induce an increase of the plasmatic Epo concentration (38), and during the present investigation the same results were confirmed with an analysis of Epo mRNA expression in the same experimental conditions. The cellular source of Epo production still remains an important goal to achieve, and the heterogenous cell population of the liver gave rise to several controversial debates still not solved (39).

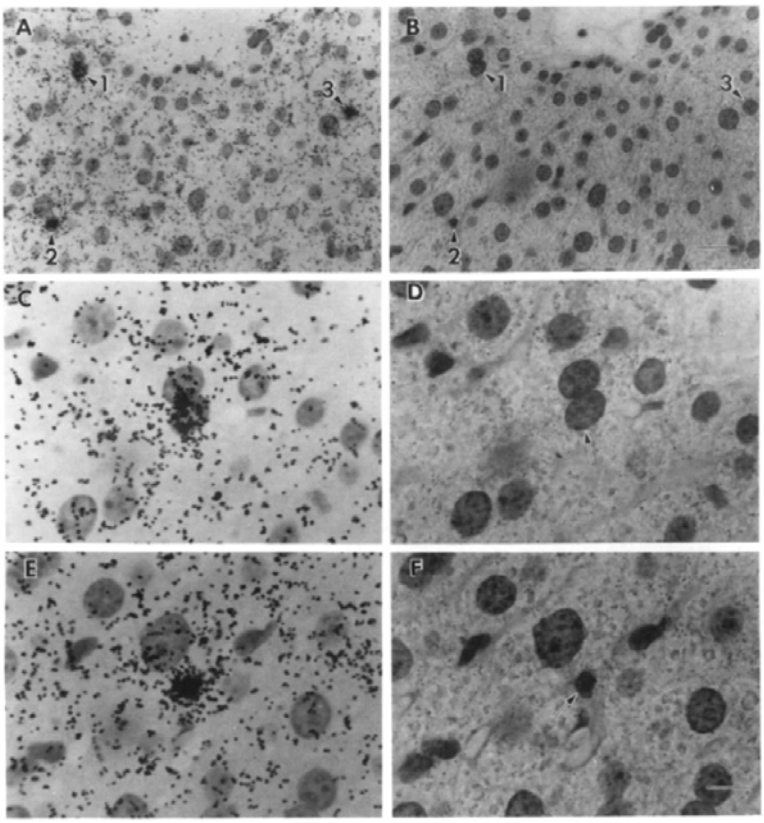



***Figure 2. Localization Epo mRNA expression in the liver by in situ hybridization (from Koury T et al, Blood 1991; 77(11): 2497-2503).*** *The authors identified two cell populations able to produce EPO in the liver: hepatocytes and a population of non-epithelial cells found in or adjacent to sinusoids. The non-epithelial cells were similar to EPO-producing interstitial cells in the renal cortex. (A-B) Low magnification showing three positive cells stained with HE and restained without silver grains. (C-D-E) Same sections at higher magnification. In (F) the cells with non-epithelial morphology are identified within a sinusoid.*

Epo expression in the central nervous system (CNS) has recently been proposed for normal brain development, but it has been reported to be stimulated by hypoxia, although with different temporal pattern (40). Epo expression has also been found in the testis and an interesting point in common with the brain is the fact that the production of Epo at these sites may be separated from the systemic circulation by the blood-testis or blood-brain barrier respectively (41). Highly sensitive mRNA detection systems have revealed EPO mRNA expression in other organs such as uterus, lung, spleen, heart and the bone marrow (42).

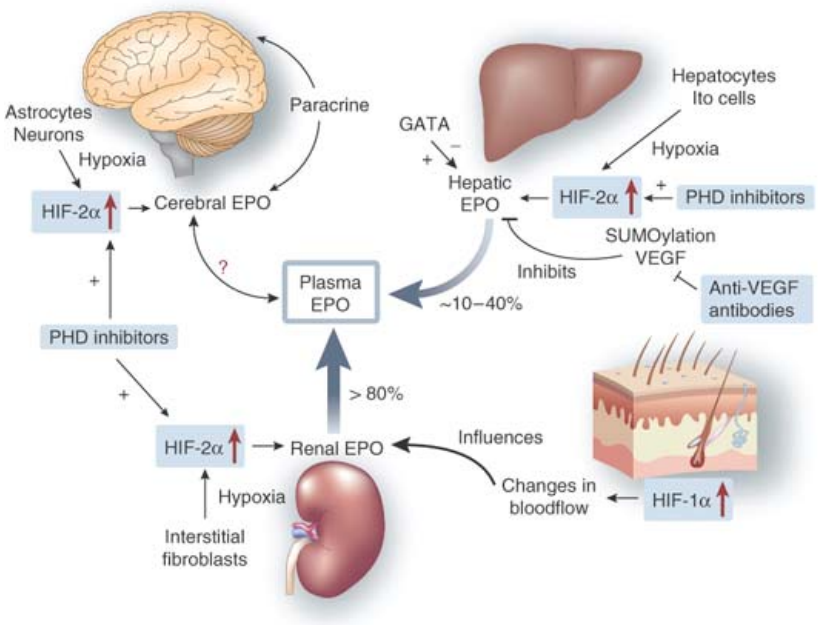



***Figure 3. Oxygen sensing in mammals (from Weidemann A and Johnson R, Kid Int 2009; 75(7): 682-688).*** *The majority of the plasma EPO in adult mammals is produced by interstitial fibroblasts in the kidney. Renal expression has been shown to be influenced by the hypoxic response of the skin, involving HIF-1α (rather than HIF-2α). Hepatic EPO is produced in Ito cells and hepatocytes, where HIF-2α is the main regulator of hypoxic EPO induction. In the central nervous system, EPO produced by astrocytes and neurons is suggested to exert paracrine effects and is not still clear if the glycoprotein can cross the intact blood-brain barrier in both ways.*

## 1.3 REGULATION OF THE ERYTHROPOIETIN GENE.

### *1.3.1 The erythropoietin gene.*

In recent times, a tissue culture model of isolated perfused kidney offered to investigators an *ex vivo* system to study the regulation of Epo. Cell lines able to produce significant amounts of Epo even in normoxic conditions were discovered by analysis of several renal and hepatic cells in culture (43). The discovery of a tissue culture model demonstrated that individual cells contain specific structures necessary for oxygen sensing and the consequent regulation of gene expression.

Tissue-specific expression of Epo gene depends on distinct upstream (5') DNA sequences and the control of the gene is regulated by several transcription factors. A liver-specific DNase I sensible site was discovered in the 3' flanking sequence of an Epo transgene model. The analysis of this region of the Epo gene by transient transfections of reporter constructs led to the identification of a hypoxically inducible enhancer (44). The enhancer demonstrated the same stimulus specificity in the induction of Epo gene with responses to hypoxia, cobaltous chloride and iron chelation. Detailed characterization of the Epo 3' enhancer defined 3 sites that are crucial for regulation by hypoxia (figure 4) (45). On the 5'side, the sequence CACGTGCT was the first response element to be characterized for the transcription factor, hypoxia inducible factor-1 (HIF-1). Binding of HIF-1 to this site is induced by hypoxia, and an intact HIF-1 binding site is necessary for hypoxically inducible function of the Epo enhancer (46). In addition to the hypoxically inducible DNA-binding activity, HIF-1, this site also binds another complex constitutively. A second site, 7bp 3' to the HIF-1 site has the sequence CACA in the human Epo gene. No protein is known to bind this site but mutation of this sequence inhibits hypoxia inducible activity of the enhancer. These first two sites require the presence of a third site for hypoxically inducible transcription; this third sequence is a direct repeat of 2 steroid hormone receptor half sites separated by 2bp (TGACCTCTTGACCC), termed a DR-2 site (47). Binding of protein to this site is not oxygen-dependent either in vivo or in vitro. Analyzing a variety of in vitro translated orphan receptors showed that HNF-4 (hepatocyte nuclear factor-4) is capable to specifically bind this site (48). Hypoxia-inducible transcription factors cooperate with HNF-4 by direct protein-protein interaction and through the recruitment of the transcriptional co-activator complex CBP/p300.

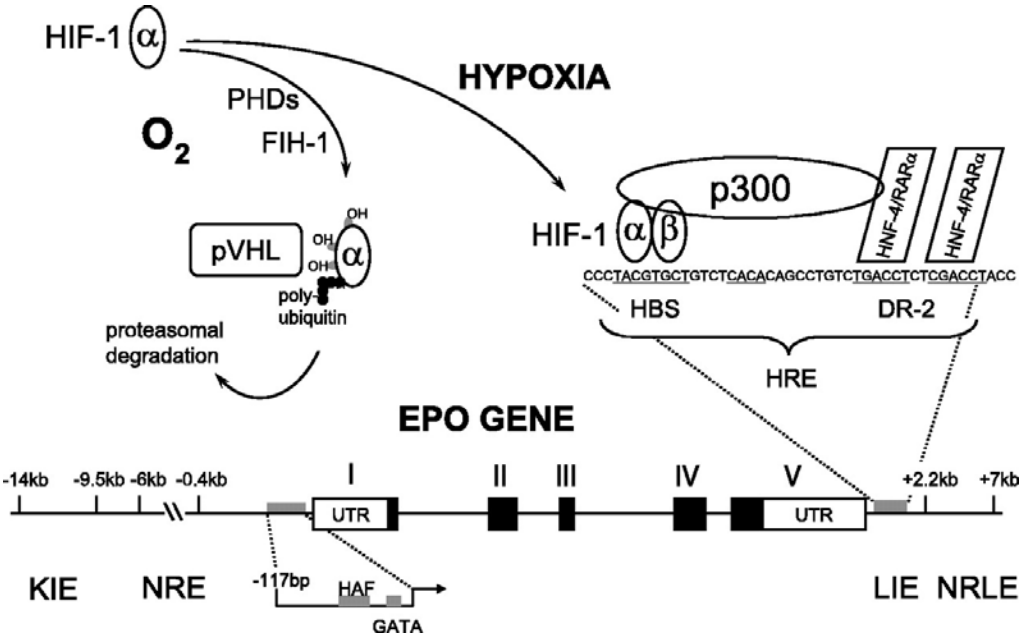



***Figure 4. Regulation of the EPO gene (from Fandrey J, AJP-Regul Integr Comp Physiol 2004; 286:R977-R988).*** *The EPO-gene regulatory elements are constituted by the kidney inducible element (KIE), the negative regulatory element (NRE), the 5'- and 3'-untranslated region (UTR), the liver-inducible element (LIE), and the negative regulatory liver element (NRLE). At higher magnification, the minimal promoter with the hypoxia associated factor and the GATA binding sites is shown.*

### *1.3.2 Molecular mechanisms of $O_2$ sensing: the HIF family.*

The discovery of HIF-1 by Semenza and co-workers has provided a clear insight into the molecular mechanisms of $O_2$ sensing (49). HIF-1 is a heterodimeric protein composed of the subunits α (HIF-1α, 120 kDa) and β (HIF-1β, 95kDa), which belong to the family of basic helix loop helix Per-ARNT-Sim proteins (bHLH-PAS). The N-terminal bHLH-PAS domains are required for dimerisation and its DNA binding properties. The C-terminus of HIF-1α comprises a transactivation domain (TAD) that can be subdivided into an N-terminal (N-TAD) and a C-terminal (C-TAD) part. The N-TAD overlaps with an $O_2$-dependent degradation domain (O-DDD). The critical residue in this domain is a proline located in position 564. Catalysed by precise prolyl-4-hydroxylases (PHD) this $Pro^{564}$ is hydroxylated in presence of $O_2$, $Fe^{2+}$ and 2-oxoglutarate. The

prolyl hydroxylated HIF-1α associates with the von Hippel-Lindau tumor suppressor protein (pVHL) to form a complex that enhances the ubiquitination by an E3 ligase and undergoes immediate proteosomal degradation (50). More recently, two bHLH-PAS HIF-α isoforms have been identified, HIF-2α (also known as endothelial PAS domain protein 1, EPAS1) and HIF-3α. HIF-2α is also $O_2$–dependent and can dimerise with HIF-1β in hypoxia but with differences concerning its tissue and cell specific mRNA expression pattern. This transcription factor was initially detected in endothelial cells, and consequently it was identified also in interstitial cells of other organs, like fibroblasts-like cells of the kidney. Accumulation of HIF-2α is predominantly due to post-translational regulation as mRNA levels are not significantly induced during hypoxia. On the other hand particularly high levels of HIF-2α mRNA are detectable in tissues that are important for the systemic exchanges of $O_2$. In contrast to HIF-1α and HIF-2α, HIF-3α lacks a transcriptional activation domain and it can behave like a regulatory subunit involved in the suppression of the hypoxia-responsive gene expression (51).

The HIF-PHDs have been shown to play a major role in the control of Epo production, because they prevent HIF-α from entering the nucleus under normoxic conditions. As PHD-2 and PHD-3 are themselves HIF-target genes, their expression increases while HIF-α l levels decline on exposure to long-term hypoxia. The binding of pVHL is mandatory for the degradation of HIF-α as demonstrated in patients with mutations of pVHL characterized by increased transcription of the Epo gene (52) (figure 4). The transcriptional activity of the HIFs is further suppressed by a third $O_2$-dependent hydroxylation, at residues $Asn^{803}$ in HIF-1α and $Asn^{851}$ in HIF-2α. This reaction is catalysed by a HIF-α specific asparaginyl hydroxylase that is known as “factor-inhibiting HIF-1” (FIH) (53). In addition to ubiquitinilation, another modification that regulates HIF-1α protein stability is covalent ligation of the small protein SUMO, which promotes the binding of VHL to HIF-1α in a hydroxylation-independent manner (54). While pVHL has been well established as the

key player in the regulation of the oxygen-dependent degradation of HIF-1α, there has been increasing evidence of pVHL-independent pathways schematically sumed up in figure 5.

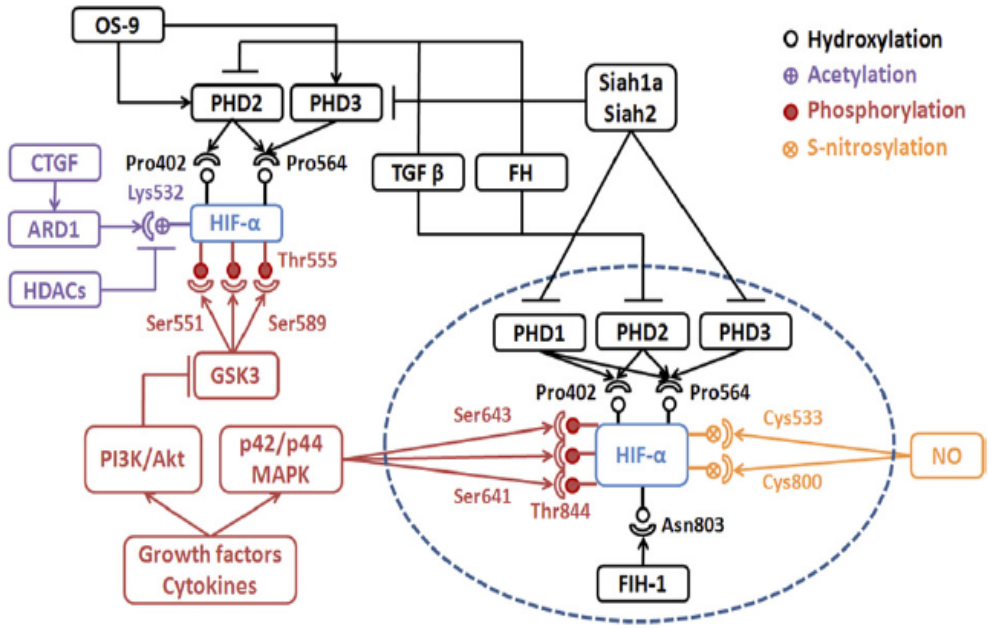



***Figure 5. HIF-1α oxygen-dependent and –independent protein regulation (from Wei W and Yu DX Cancer Lett 2007; 257:145-156).*** *Under normoxic conditions HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) enhancing the recruitment of the pVHL E3 ligase complex to HIF-1, a process facilitated by SSAT2 (spermidine/spermine-N-acetyltransferase) which binds to HIF-1, pVHL and elongin C. The pVHL E3 ligase complex ubiquitylases HIF-1α leading to its degradation. During hypoxia HIF-1α undergoes SUMOylation which can facilitate the recognition of HIF-1 by the pVHL E3 ligase complex and lead to HIF-1α degradation. HIF-1α SUMOylation can be reversed by SENP1 (SUMO1/sentrin specific peptidase1), resulting in stabilization. Hypoxia induced RSUME (RWD-containing SUMOylation enhancer) can increase HIF-1 stability. Other oxygen-independent regulator of HIF-1 include GSK3 (glycogen synthase kinase 3) which phosphorylate HIF-1α leading to its ubiquitylation and RACK1 which binds to HIF-1 as a dimer when HSP90 is inhibited and recruits the components of the E3 ligase complex through a process facilitated by SSAT1. The cytokine TGF-β and fumarate hydratase (FH) can inhibit hydroxylation process. Acetylation of HIF-1α induces its destabilization, whereas deacytilation operated by histone deacetylases (HDAC) prevents its degradation. S-nitrosylation, a redox-related modification of cystein thiol by nitric oxide (NO), also contribute to HIF-1α protein stabilization.*

Semenza and co-workers identifying HIF-1α as a regulator of the HRE (hypoxia responsive element) on the EPO promoter, described a wide number of hypoxia responsive genes under control of this transcription factor. In fact, other then controlling Epo and Vegf in specific cell populations, HIF-1α has also been implicated in transcriptional activation of the genes encoding iNOS and heme oxygenase-1, which are responsible for production of the vasoactive molecules NO and CO, respectively (55). Thus, HIF-1 plays a role in both the production of erythrocytes and of capillaries through which they transport $O_2$ to the tissues. HIF-1α also mediates a critical intracellular physiologic response to hypoxia: the increased rate of glycolisis in hypoxic cells. Specific proteins regulated by HIF-1 include the glucose transporters GLUT1 and GLUT3 and the glycolytic enzymes aldolase A, aldolase C, ENO1, lactate dehydrogenase A, phosphofruktokinase L, phosphoglycerate kinase 1, and pyruvate kinase M (56).

With regard to HIF-2α targets, transcriptional induction of the angiopoietin-1-receptor (Tie-2) is highly linked to HIF-2 activity, possibly due to a predominance of HIF-2α over HIF-1α expression in vascular endothelial cells (57). Similarly, the VEGF receptor-2 (KDR) gene in regulated in a HIF-2α-specific manner, which has been associated with endothelial cell-specific transcription, as well as a result of cooperation between the ETS-1 transcription factor and HIF-2α, but not HIF-1α, within the KDR promoter (58). Additional genes that are increasingly appreciated as HIF-2α-predominant targets include VEGF, transforming growth factor-α (TGFA), EPO, membrane type-1 matrix metalloproteinase (MT-MMP) and lysyl oxidase (LOX) (59).

## 1.4 LIVER AND ACUTE PHASE RESPONSE.

### *1.4.1 Acute phase reaction: mediators and target.*

The acute-phase response comprises the nonspecific physiological and biochemical responses of endothermic animals to most forms of tissue damage, infection, inflammation and malignant

neoplasia. A large number of changes, distant from the site or sites of inflammation and involving many organ systems, may accompany the reaction. These changes may be divided into alterations in the concentrations of many plasma proteins known as acute phase proteins and a large number of behavioural, physiological, biochemical and nutritional changes (60). An acute phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase protein) by at least 25 percent during inflammatory disorders. The changes in the concentrations of acute phase-proteins are due largely to changes in their production on hepatocytes. Although the concentrations of multiple components of the acute-phase response commonly increase together, not all of them increase uniformly in all patients with the same illness. These variations, which indicate that the components of the acute-phase response are individually regulated, may be explained in part by differences in the patterns of production of specific cytokines or their modulators in different path physiologic states. The various acute phase proteins differ markedly in the rise or decline of their plasma levels and also in their final concentrations, but they have at least one common function: they are engaged in various physiological processes which tend to restore the delicate homeostatic balance disturbed by the injury, tissue necrosis or infection (61).

Liver response to injury characterizes the central phase of the acute-phase reaction but it is intimately linked to other adaptive and compensative measures as fever, leukocytosis and enhanced protein degradation in muscles. All these mechanisms are anyway regulated by mediators synthesized and released by hepatocytes. Fever is representative of the neuroendocrine changes that characterize the acute-phase response. Although several cytokines may induce fever, interleukin-6 produced in the brain stem is required for the final steps leading to fever. Other neuroendocrine changes reflect complex interactions among cytokines; the hypothalamic-pituitary-adrenal axis for example is responsible of the production of corticotrophin and cortisol production during acute-phase response. The behavioural changes that often accompany inflammation including anorexia, somnolence, and lethargy are similarly induced by cytokines. Cachexia, the loss of body mass that

occurs in prolonged inflammatory diseases, results from decreases in skeletal muscle, fat tissue and bone mass. Also in this case, cytokines represent the major inducer.

Interleukin-8 also, identified as an acute-phase mediator massively released by hepatocytes, regulates the migration of granulocytes from the bone marrow in the sites of injury responsible of the increased leukocytosis. Inflammation-associated cytokines also alter many intracellular hepatic constituents, including inducible nitric oxide, manganese superoxide dismutase and microsomal heme oxygenase. Moreover, the systemic, endocrine and metabolic, response to acute inflammation includes in this way the release of hormones which induces catabolism and gluconeogenesis such as glucagon, insulin, adrenocorticotropic hormone, growth hormone, thyroxin and catecholamines (62).

**TABLE 1.** HUMAN ACUTE-PHASE PROTEINS.

| |
|---|
| **Proteins whose plasma concentrations increase** |
| Complement system |
| C3 |
| C4 |
| C9 |
| Factor B |
| C1 inhibitor |
| C4b-binding protein |
| Mannose-binding lectin |
| Coagulation and fibrinolytic system |
| Fibrinogen |
| Plasminogen |
| Tissue plasminogen activator |
| Urokinase |
| Protein S |
| Vitronectin |
| Plasminogen-activator inhibitor 1 |
| Antiproteases |
| $\alpha_1$-Protease inhibitor |
| $\alpha_1$-Antichymotrypsin |
| Pancreatic secretory trypsin inhibitor |
| Inter-$\alpha$-trypsin inhibitors |
| Transport proteins |
| Ceruloplasmin |
| Haptoglobin |
| Hemopexin |
| Participants in inflammatory responses |
| Secreted phospholipase $A_2$ |
| Lipopolysaccharide-binding protein |
| Interleukin-1–receptor antagonist |
| Granulocyte colony-stimulating factor |
| Others |
| C-reactive protein |
| Serum amyloid A |
| $\alpha_1$-Acid glycoprotein |
| Fibronectin |
| Ferritin |
| Angiotensinogen |
| **Proteins whose plasma concentrations decrease** |
| Albumin |
| Transferrin |
| Transthyretin |
| $\alpha_2$-HS glycoprotein |
| Alpha-fetoprotein |
| Thyroxine-binding globulin |
| Insulin-like growth factor I |
| Factor XII |

***Table 1. Principal human positively and negatively regulated acute-phase proteins (from Gabay C and Kushner I, N Eng J Med 1999; 340 (6):448-454.***

### *1.4.2 Liver and the cytokines.*

As described above, during acute phase response the synthesis of a number of proteins is rapidly up-regulated, principally in hepatocytes, under the control of cytokines originating at the site of pathology. One major part of the acute-phase reaction is focused on the liver, due to the fact that this organ is a prominent source as well as a target of cytokines (63). Macrophages, which after activation are generally considered to be the main source of inflammatory cytokines and mediators (e.g. reactive oxygen species and nitric oxide), are present within the liver. Kupffer cells lining the hepatic sinusoids are the largest population of resident tissue macrophages of the body. In a second line, hepatocytes are targets of cytokines of the acute phase response and which synthesize the majority of plasma proteins (64). Cytokines are released from activated Kupffer cells directly into the blood and are likely to induce the acute-phase in any other organ. Furthermore, Kupffer cells-derived cytokines act in a paracrine manner on protein synthesis of hepatocytes.

The acute-phase cytokines can be classified according to their functions into two major groups. IL-1-type cytokines include IL-1α, IL-1β, TNF-α and TNF-β. They stimulate the synthesis of some of the positive acute-phase proteins, such as C-reactive protein, serum amyloid A and haemopexin, and inhibit negative APPs such as albumin. IL-6-type cytokines (IL-6, IL-11, LIF, CNTF) stimulate type-2 acute-phase proteins, such as fibrinogen, α-1-antitrypsin, haptoglobin and ceruloplasmin.

Hepatocytes bear a variety of cytokines receptors. Receptors for the mentioned acute-phase cytokines may gain control over synthesis of plasma protein that are produced by hepatocytes and most of the released cytokines may stimulate hepatocytes and other non-parenchymal cells in a paracrine manner.

Besides the ubiquitous transcription factors involved in the basal transcription machinery of most cell types and the well-known NF-kB and AP-1 factors, the hepatocytes contains further transcription factors that are prominent in only a limited number of cell types. These factors mostly include some members of the Hepatocyte Nuclear Factor (HNF)-1, HNF-3, HNF-4, HNF-6, CAAT/enhancer binding protein (C/EBP) and Signal Transducer and Activator of Transcription

(STAT) families. Various stimuli that promote or mimic an APR have been shown to down-regulate the amount and/or the activity of some liver-enriched transcription factors and simultaneously up-regulate others. Specifically, most of the genes coding for liver-enriched transcription factors are prone to APR-associated, transcriptional regulations while many of them also undergo post-transcriptional regulations. A further layer of complexity is added by co-factors that may enhance or inhibit transcriptional activity of other proteins in an acute-phase response-dependent manner (65). Finally, an ever growing complexity in the interplay between factors and binding sites has emerged from further studies since some promoters turned out to share numerous binding sites for a wealth of acute-phase response-inducible transcription factors whose functions and possible interactions are not always fully understood.

### *1.4.3 Aseptic acute phase and sepsis: two ways for a common response.*

The intra-muscular injection of turpentine oil induces a local tissue damage that is responsible for the development of a systemic acute-phase response. This experimental model has been well characterized to reproduce the conditions of a sterile inflammation, in which an inflammatory response develops in absence of any microbial stimulus. An important role for IL-1 in the induction of most of the metabolic changes observed has been demonstrated with the use of anti-IL-1 receptor type I antibodies (66). In this model of localized tissue damage, a specific induction of IL-1β and IL-6 is present, without any detectable IL-1α or TNF-α production. This suggests the existence of a common cascade of cytokine production, characteristic of sterile inflammation, where IL-1β and IL-6 might play a critical role. As IL-6 has been recognized as the major cytokine involved in muscular wasting and degradation after strenuous exercise and damage, the role of this cytokine in the degradation of tissue muscle mass after turpentine oil injection has been well investigated. In TO-induced muscle degeneration, the activities of the lysosomal cathepsins B and L in the muscle begin to rise at 12h after the injection, when the infiltration of macrophages has not yet become

evident. The activities markedly increased after 1 day, together with massive infiltration of macrophages and atrophy of myofibers was observed (67). Strong expression of IL-6 mRNA was found at 12h in the injected muscle and the serum IL-6 concentration increased, remaining high until 3 days. IL-6 in the muscle induces activation of lysosomal cathepsins and consequently enhances autodigestion of myofibrils that are successively phagocytised by the infiltrating macrophages with the formation of a sterile abscess. In the IL-6 knockout mice the activities of cathepsins (B and B+L) in the injected muscle were high as the control mice, suggesting compensatory mechanisms for lysosomal cathepsins from other cytokines. This compensatory mechanisms in absence of IL-6 that drives to a different inflammatory environment in the injured muscle, induces in this model also a different temporal pattern and qualitative modifications in the expression of acute-phase mediators.

Toll-like receptors (TLRs) are important initiators of innate immunity, recognizing diverse microbial products which are collectively known as pathogen-associated molecular patterns (PAMPs). The best characterized TLR is TLR4 which recognises the Gram-negative product lipopolysaccharide (LPS). Once activated, a TLR trigger a cascade of cellular signals, culminating in the eventual activation of NF-kB which binds to a discrete nucleotide sequence in the up-stream regions of genes that produces acute-phase cytokines thereby regulating their expression (68). Upon triggering, TLR4 signalling drives Kupffer cells to produce TNF-α, IL-1β, IL-6, IL-12, IL-18 and IL-10. Hepatocytes may up-take and eliminate endotoxin from portal and systemic circulation although *in vivo* hepatocytes response through TLR4 is fairly weak. Hepatic stellate cells (HSCs) located in the space of Disse have been shown to express TLR4 as well as biliary epithelial cells and liver sinusoidal endothelial cells (69). IL-1 has been considered one of the principal mediators of LPS-induced toxicity but other mediators such as TNF-α, have been shown to be critical for the response to LPS. IL-1 and TNF reciprocally induce each other and are strongly synergistic (70). The response to LPS has been studied in most of the cytokine-related knockout mice generated to date. IL-6-deficient mice show only a moderately alteration of the acute-phase response following

endotoxin administration, as well as TNF-R-deficient mice which exhibits a response almost comparable to the wild-type animals (71).

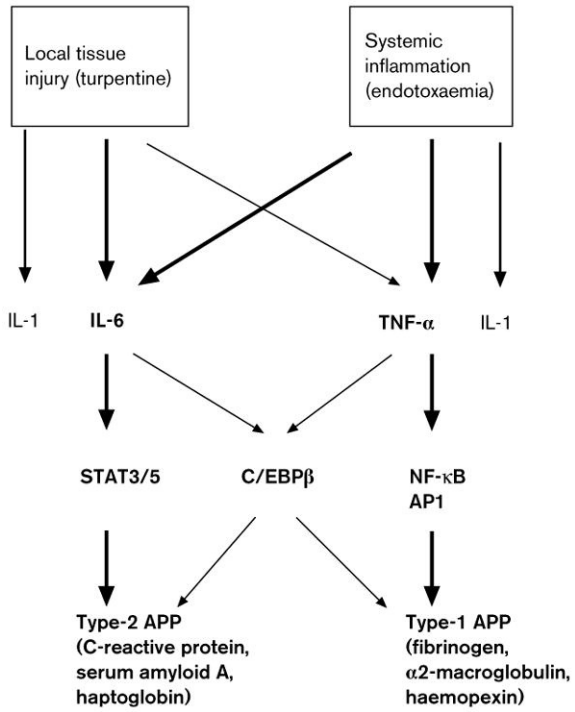



***Figure 6. Signalling pathways interaction and transcription factors activated by different acute-phase cytokines regulating the development of the acute-phase reaction (from Ramadori G and Armbrust T, Eur J Gastroent Hep 2001; 13:777-784).***

### *1.4.4 Iron mobilization during acute phase reaction.*

The liver is the central organ of iron metabolism. It receives the iron contained in the heme of the erythrocytes which are eliminated by the Kupffer cells. The hepatocyte represents the major iron-storing cells of the body. It expresses both classic transferrin receptors (TfR1 and TfR2) and is thought to possess ferritin receptors. Upon binding the Transferrin (Tf)-TfR-complex is internalized into endosomes where the Fe is then released from Tf by a reduction of endosomal pH. From this point iron can be used for a variety of metabolic processes or stored within the protein ferritin. Iron is delivered to the bone marrow primarly on the transport protein transferrin, with some

contribution by other iron-carrier proteins. At the bone marrow, the dominant regulatory protein that control synthesis of new red body cells (RBC) is erythropoietin, which maintains production at about 20 ml of new RBC/day in the healthy state. As previously discussed, Epo acts at the bone marrow by preventing apoptosis of blasts cells, so that erythrocytes precursors are able to maintain growth and division, and develop to become reticulocytes and then mature RBC. Iron is released to transferrin through a transmembrane protein on storage cells called ferroportin. Through mechanisms not yet fully understood, storage iron held in ferritin is released to ferroportin, passes out the cells and is picked up at the surface of the storage cell by transferrin. Inflammation and the acute phase response interact with iron metabolism at several levels. These physio-pathological conditions reduce the serum concentration of iron through an increase of iron storage and an inhibition of iron release. A key cytokine in this context is IL-6 that acts to increase the production of hepcidin in the liver, although other cytokines has been shown to contribute to the activation of hepcidin synthesis (72). Hepcidin then promotes the internalization and degradation of ferroportin, and as a consequence the export of iron is inhibited. Moreover, important changes in genes involved in iron metabolism have been described during acute-phase reaction (73).

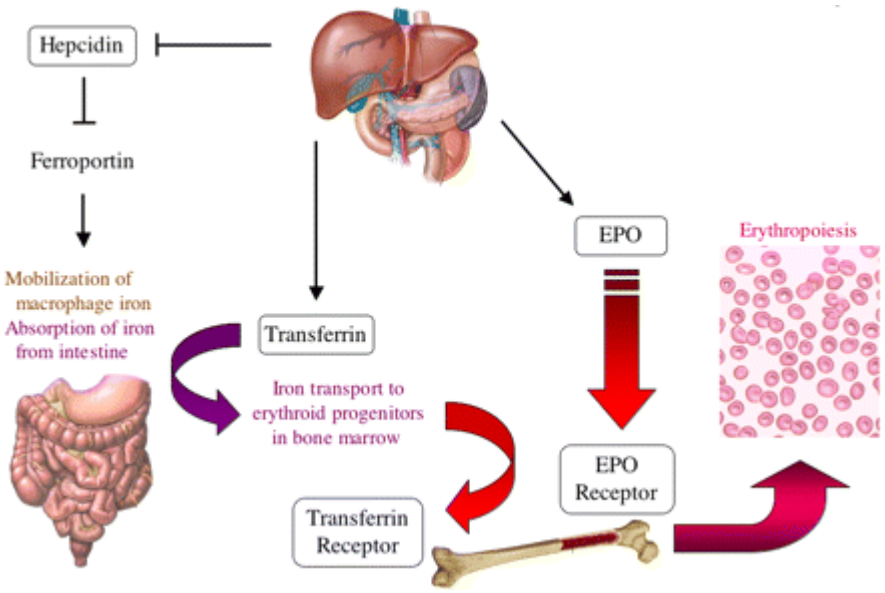



***Figure 7. HIF-1 regulates the expression of multiple genes to stimulate erythropoiesis in response to hypoxia (from Semenza GL, Blood 2009, 114 (10):2015-2019).*** *Erythropoiesis involves uptake by the bone marrow of large amounts of iron, which are used in the synthesis of haemoglobin. In the liver, HIF-1 stimulates iron uptake by repressing the gene encoding hepcidin, which is an inhibitor of ferroportin, the major protein responsible for intestinal iron up-take. HIF-1 also activates hepatic synthesis of transferrin, the major plasma protein responsible for transporting iron from the intestine to the bone marrow via transferrin receptor. HIF-1 in this way directly regulates the expression of 5 gene products (EPO, EPOR, hepcidin, transferrin, and transferrin receptor) involving 5 different organs (kidney, liver, intestine, blood and bone marrow) to control erythropoiesis.*

### *1.4.5 Inflammation and EPO-gene regulation.*

The anaemia of inflammation has received intense studies in the last 20 years. A striking paradox is that total body iron stores can be very high, but there is still restricted iron available for erythropoiesis. This form of anaemia is typically first identified as a failure to respond to iron supplementation. Erythropoietin elevation may be less than the level predicted in proportion to the anaemia: in iron-deficiency anaemia, an Hb of 10 g/dl would lead to Epo of 150-200 mU/ml (from normal of 15mU/ml), but in anaemia of inflammation Epo may only increase to 50-75 mU/ml (74). Although many clinical and experimental studies about Epo production during acute inflammation

and acute phase response reported controversial results, the suppressive effects on erythropoiesis observed in this situation might be primarily due to alterations in sensitivity to erythropoietin (75). The effective regulation of Epo-gene during acute phase response has not been sufficiently analyzed until today and the site of production and mechanisms involved in its regulation required further investigations for the comprehension of the potential role of Epo during inflammation. This is the critical point proposed in the present study in order to offer a detailed analysis of this physio-pathological situation.

Furthermore, surgical trauma or tissue injury induce a state of inflammation with an acute-phase reaction and anaemia. The injury response represents a complex and very well orchestrated process organized in distinct stages. The first component of this cascade is initiated by immune-competent cells recognizing characteristic molecular signatures of the pathogen or of the injured tissue. Results of several studies have shown that in this setting, the biological effects of TNF-α and Epo are antagonistic (76). The ambient tissue balance of these opposing molecules determine whether inflammation or tissue protection dominates. It is in this environment that the paracrine release of EPO might act to limit the collateral damage initiated by the tissue injury response.

It is also due to mention that foci of inflammation are characterized by low levels of oxygen and glucose, together with high concentrations of lactate and reductive metabolites, including free oxygen radicals. To maintain energy homeostasis and carry out their biological activities in this specialized environment, phagocytic cell types must generate ATP via glycolysis. Because up-regulation of almost every enzyme in the glycolytic pathway is mediated almost exclusively by HIF-1α, a role for this transcription factor in supporting phagocyte function during inflammation has been suggested. The contribution of HIF-1α to myeloid cell-mediated inflammatory pathologies prompted immediate consideration of the potential of the transcription factor to mediate central functions of macrophages and neutrophils in innate host defence. Whereas in healthy tissues, oxygen concentration is among 2.5-9%, much lower levels are present in wound and sites of infection. The deletion of HIF-1α has been shown not to alter phagocyte production of reactive

oxygen species through the respiratory burst, but the expression of a number of other molecular inflammatory mediators belonging to the host innate defence was significantly correlated to HIF-1α levels. HIF-1α role in the innate immune response has emerged as a crucial regulator since it has been recognized in the liver as regulator of the production of the peptide hepcidin, promoting iron absorption and mobilization, and stimulates red blood cell synthesis by increasing EPO production. Its activation in hepatocytes during acute-phase response delineates a novel functional role of HIF-1α in the regulation of this complex physio-pathological process.

# 2 MATERIALS AND METHODS.

## 2.1 MATERIALS.

All the chemicals used were of analytical grade and were purchased from commercial sources as indicated below: TRIZOL reagent (Invitrogen GmbH, Karlsruhe, Germany) for isolation of RNA from the tissues; real-time polymerase chain reaction (PCR) primers and primers for northern blot from Invitrogen, M-MLV reverse transcriptase, reverse transcription buffer and 0.1M DTT, platinum Sybr green qPCR-UDG mix from Invitrogen, dNTPs, protector RNase inhibitor, bovine insulin, Klenow enzyme, primer oligo(DT)15 for cDNA synthesis and salmon sperm DNA from Roche (Mannheim, Germany); α-32P-labelled deoxycytidine triphosphate (specific activity 3000 Ci/mmol), NICK TM columns and Hybond N nylon membranes from Amersham Pharmacia Biotech (Freiburg, Germany); hybridization solution QuickHyb from Stratagene (Germany), iron ferrozine from Rolf Greiner BioChemica (Flacht, Germany) and Quantikine enzyme linked immunosorbent assay (ELISA) kits from R&D systems (anti-rat and mouse EPO). All other reagents and chemicals were from Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany). Mouse anti-human/rat EPO antibody from Acris Antibodies GmbH (Hiddenhausen, Germany) and rabbit anti-mouse EPO from Santa Cruz Biotech , rabbit anti-HIF-1α and anti-HIF-2α from Novus Biological (Cambridge, UK), anti-beta-actin antibody from Sigma Aldrich (Germany), rat anti-mouse F4/80 from AbD Serotech (Germany), secondary and antibodies anti-mouse and anti-rabbit IgGs from DAKO (Germany).

## 2.2 ANIMALS.

For the first part of the study, male Wistar rats of about 170–200 g body weight were purchased from Harlan-Winkelmann (Brochen, Germany). The animals were kept under standard conditions with 12 h light/dark cycles and ad libitum access to fresh water and food pellets. All animals were cared for according to the University's guidelines, the German convention for the protection of animals and NIH guidelines.

In the second part adult male B6.129S2-*Il6*$^{tm1Kopf}$ (IL6-knockout) mice and control wild type adult male C57BL/6J mice (25 – 28 g) were purchased from Jackson Laboratory. The animals were kept under standard conditions with 12 h light/dark cycles and ad libitum access to fresh water and food pellets. All animals were cared for according to the University's guidelines, the German convention for the protection of animals and NIH guidelines.

### *2.2.1 Experimental models of acute-phase reaction.*

APR was induced in ether-anesthetized rats by intramuscular injection of 5 ml/kg TO in both right and left hind limbs (n=4). Control animals did not receive any injections (n=4).

Injected and non-injected control rats were euthanized 0.5h, 1h, 2h, 4h, 6h, 12h, 24h, 36h and 48h after TO injection under pentobarbital anaesthesia. Liver, kidney, heart, lungs, spleen and the injured muscle were excised and minced, rinsed with physiological sodium saline, snap frozen in liquid nitrogen and stored at -80°C till further use. Blood samples were collected from the inferior vena cava of the control and treated animals, allowed to clot overnight at 4°C and centrifuged for 20 min at 2000 g. Serum was removed and stored at -20°C.

For the mice experiments, a group of animals (n=3) for each strain, control (C57BL/6J) and IL6-knockout, was injected intramuscularly with 0.1 ml TO in both right and left hind limbs to induce an aseptic acute phase reaction. A second group of animals (n=3) for each strain was injected intraperitoneously with 50 μg LPS from E.Coli serum type to induce a non-sterile acute phase

reaction. Animals were euthanized 2h, 4h, 6h, 12h and 24h after the treatments under pentobarbital anaesthesia. Liver, kidney and injured muscle tissues were excised and minced, rinsed with physiological sodium saline, snap frozen in liquid nitrogen and stored at -80°C till further use. Blood samples were collected from the inferior vena cava of the control and treated animals, allowed to clot overnight at 4°C and centrifuged for 20 min at 2000 g. Serum was removed and stored at -20°C.

## 2.3 GENE-EXPRESSION ANALYSIS.

### *2.3.1 RNA Isolation and Quantitative Real-Time PCR.*

During the whole study, total RNA was isolated from liver, kidney, heart, lung, spleen and injured muscle tissue samples with TRIzol reagent according to the manufacturer's instructions. Briefly, tissue samples were homogenized in 1 ml TRIzol reagent per 100 mg of tissue using a power homogenizer. After 5 minutes incubation at room temperature, 0.2 ml chloroform per sample was added and samples were vigorously shaked and incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000 g for 30 minutes and the acqueous phase so obtained in the supernatants was carefully collected. A volume of 0.5 ml of isopropyl alcohol was succevssively added to the collected phase and samples were incubated at room temperature for 10 minutes. The RNA precipitation was favoured centrifuging again at 12,000 g for 10 minutes. After a short washing step in ethanol 75%, RNA was shortly air-dried and resuspended in a volume of approximately 50 µl of RNase and Dnase free water. The RNA was then quantified by measuring the absorbance at 260nm/280nm.

The cDNA was generated by reverse transcription of 3.0 µg of total RNA with 100nM of dNTPs, 50pM of primer oligo(dT)15, 200U of moloney murine leukemia virus reverse transcriptase (M-MLV RT), 16U of protector RNase inhibitor, 1X RT buffer and 2.5 ml of 0.1M DTT for 1 h at 40°C. The primers used in the work are listed in Table I for rats and Table II for mice were

designed using the software PerlPrimer v1.1.17. Gene expression was analyzed using Fast SYBR Green Master Mix (Applied Byosystem) was performed through two-step cycling (95–60°C) for 45 cycles in an StepOne Plus RT-PCR detection system (Applied Biosystem), following the instructions of the supplier. Beta-actin and ubiquitin c were used as housekeeping genes. All samples were assayed in duplicate. The results were normalized to the endogenous controls (β-actin and Ubiquitin C), and fold change of the gene expression was calculated using threshold cycle (Ct) values.

**Table I. Rat primers used in the study**

| Gene | Forward | Reverse |
|---|---|---|
| rat EPO | CCAGCCACCAGAGAGTCTTC | TGCAGAAAGTATCCGCTGTG |
| rat HIF-1α | ACAAGAAACCGCCTATGACG | TAAATTGAACGGCCCAAAAG |
| rat HIF-2α | TGACTTCCTCATCCTTGCGACCA | ATTCATAGGCAGAGCGGCCAAGTA |
| rat UBC | CACCAAGAACGTCAAACAGGAA | AAGACACCTCCCCATCAA ACC |
| rat β-ACTIN | TGTCACCAACTGGGACGATA | AACACAGCCTGGATGGCTAC |
| rat IL-6 | GTCAACTCCATCTGCCCTTCAG | GGCAGTGGCTGTCAACAACAT |
| rat IL-1β | TACCTATGTCTTGCCCGTGGAG | ATCATCCCACGAGTCACAGAGG |

**Table II. Mouse primers used in the study.**

| Gene | Forward | Reverse |
|---|---|---|
| Mouse IL-6 | TTCCATCCAGTTGCCTTCTTGG | TTCTCATTTCCACGATTTCCCAG |
| Mouse IL-1β | TACAGGCTCCGAGATGAACA | AGGCCACAGGTATTTTGTCG |
| Mouse TNF-α | CAAACCACCAAGTGGAGGAG | GTGGGTGAGGAGCACGTAGT |
| Mouse EPO | ATGTCACGATGGGTTGTG | GGAGGAATTGGCTAGCAG |
| Mouse HIF-1α | TCCATCTTCTACCCAAGTACC | GCAATAATGTTCCAATTCCT |
| Mouse HIF-2α | GAGCGTGACTTCTTCATGAG | CAGTTCCAAGATTCTGTCGT |
| Mouse SOD-2 | GCACTGAAGTTCAATGGTG | GAATAAGGCCTGTTGTTCCT |
| Mouse β-actin | ATTGTTACCAACTGGGACGACATG | CGAAGTCTAGAGCAACATAGCACA |

### *2.3.2 Northern blot analysis of the acute-phase protein serum amyloid A2.*

In order to verify the effective induction of acute-phase reaction, a Northern hybridization for one of the major acute-phase protein serum amyloid 2 (SAA2) was performed in hepatic RNA extracted from wild type and IL6-knockout mice. Each RNA sample (10 μg of total RNA) in a volume not more than 10 μl was mixed with 7.5 μl of sample buffer. RNA probes mixed with sample buffer were denatured by heating at 65°C for 10 min. After a short cooling on ice, each sample was mixed with 3 μl of loading buffer and loaded into a 1% formaldehyde agarose gel. The electrophoresis was performed at constant voltage of 80 V for 1-1.5 h. After electrophoresis, the quality of RNA was estimated under UV transilluminator built-in Eagle Eye™ system (Stratagene); the gel was photographed, and the procedure was immediately continued to blotting. The gel was transferred overnight by capillary transfer in 20x SSC (buffer solution with 175.3 g NaCl + 88.2 g sodium citrate, pH 7.4) and successively the RNA was fixed on the menbrane by UV crosslinking for 2 min from both sides using Stratalinker™ 180 (Stratagene) set. The prehybridization which is necessary

to prevent unspecific binding was performed for 1 h with 10 ml of QuikHyb hybridization solution (Amersham) at 68°C in a hybridization oven. Radiolabeled probe was mixed with double volume of salmon sperm DNA and afterwards denatured for 5 min at 95°C. The hybridization was carried out for 1 h at 68°C in a hybridization oven. The probe was synthesized by PCR reaction with a cDNA obtained form rat liver with the use of the following primers for murine SAA2: fw-GCTGGCTGGAAAGATGGA; rv-ATTTGGCAGGCAGTCCAG. The cDNA (1μg) probe was radiolabelled with α-$P^{32}$[CTP] using Nick translation system with a DNase I/Polymerase I mix (Invitrogen) for 90 minutes at about 16°C

### *2.3.3 Southern blot analysis of PCR products.*

In order to confirm the primers specificity and because of the low expression of the gene in normoxic conditions, EPO gene-expression was also analyzed in the liver and the kidney as previously published (77) by conventional PCR according to the following cycles: 94°C for 2 minutes, 94°C for 30 seconds, 60°C for 1 minute (40 cycles) and finally at 72°C for 2 minutes. The PCR reaction products in a volume of 10 μl for EPO and 6 μl for β-actin were separated electrophoretically in a 1.2 % agarose gel and blotted onto a nylon membrane by capillary transfer and successively crosslinked by UV. The blot was hybridized with a probe synthesized by PCR reaction with cDNA obtained from rat kidney and radiolabelled with γ-CTP through Nick translation kit. The hybridization was performed at 62°C for 2 hours, followed by washing steps with 2X SSC+0.1%SDS. β-actin cDNA was used as an internal standard.

## 2.4 BIOCHEMICAL ANALYSIS.

### *2.4.1 Proteins Isolation and Western Blot.*

For semi-quantitative analysis of protein expression western blot was performed and total protein extraction was prepared from liver samples as previously described (78).

Briefly, about 100 mg of frozen tissue was homogenized with an Ultra-Turrax TP 18/10 three times for 10 s each in 10 volumes of 50mM Tris-HCl buffer, pH 7.4, containing 150mM sodium chloride, 1mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1mM phenylmethanesulfonyl fluoride (PMSF), 1mM benzamidine, 1 mg/ml leupeptin, 10mM chymostatin, 1 mg/ml antipain and 1 mg/ml pepstatin A, with a final addiction of a phosphatise inhibitors cocktail. Samples were then centrifuged twice for 15 minutes at 10000 g. A second purification centrifugation was performed as above in order to pellet further cell debris or homogenate impurities. Protein concentration was determined with Coomassie Blue Reagent (Pierce Biotech.) and the absorbance was read with a spectrophotometer at 595 nm wavelength.

Fifty micrograms from the total protein lysate were loaded in a 4-12% Nu-PAGE Bis-Tris (Invitrogen) gel and separated after 2 hrs electrophoresis at 80V. After the transfer in a semidry apparatus at 26V for 1.5h, the membranes were blocked in 5% milk, and blotted with mouse anti-human/rat EPO (Acris GmbH, Germany) diluted 1:200, rabbit polyclonal anti-HIF-1 alpha (Novus Biological, UK) 1:400, mouse monoclonal anti-HIF-2 alpha (Novus Biological) 1:400 overnight at 4°C, and monoclonal mouse anti-β-actin (Sigma-Aldrich, Germany) 1:2000 for 2h at room temperature. The secondary antibodies were swine anti-rabbit and rabbit anti-mouse immunoglobulins (DAKO) diluted at 1:1000. Membranes were developed with ECL chemiluminescence Kit (Amersham).

### *2.4.2 Enzyme-Linked Immunoadsorbent Assay.*

Quantikine Mouse/Rat Erythropoietin ELISA kits from R&D Systems were used for detection of EPO and IL-6 concentration in serum. Serum samples and total liver homogenates (prepared according to the previous protocol) were processed according to the manufacturer's instructions. A microplate is pre-coated with monoclonal antibody specific for rat/mouse interleukin-6 or erythropoietin. Samples are pipetted into the wells and any antigen present is bound by immobilized antibody. After washing away any unbound material, an enzyme-linked polyclonal antibody specific for rat/mouse IL-6 or Epo is added. Any unbound antibody-enzyme reagent is washed away, and a substrate solution is added to the wells. The enzymatic reaction yields a blue-colored product that turns yellow when the stop solution is added. The intensity of the color measured colorimetrically is proportional to the amount of rat/mouse antigen bound in the initial step. The optical density was analyzed using a microplate reader set to dual wavelenght mode (450nm/570nm). Using the commercial kit, the detection limit in rat serum is about 8.5 pg/mL and in normal rats no EPO concentration was detectable.

### *2.4.3 Nuclear protein extracts isolation and electrophoretic mobility shift assay.*

Nuclear protein extracts from rat liver were prepared as previously described (79). Briefly, fresh liver tissues were washed in cold PBS and homogenated in 4 volumes of buffer A (10mM Hepes-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.4 mM PMSF, 1 mM $NaVO_3$, 2μg/ml pepstatin and 2μg/ml apoprotinin) with a Potter homogenizer. Liver homogenates were then centrifuged at 2300 RPM for 10 minutes and the pellet resuspended in 3 volumes of buffer A; it was then centrifuged again at 14500 RPM for 20 minutes. The nuclear pellet was resuspended in buffer C (20 mM Hepes-KOH, pH7.9, 1.5 mM $MgCl_2$, 0.42 mM NaCl, 0,2 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.4 mM PMSF, 1 mM $NaVO_3$, pepstatin and apoprotinin as above) and incubated at 4°C

for minutes with gentle agitation. Nuclear debris was pelleted by centrifugation at 14500 RPM for 30 minutes and supernatant-containing nuclear proteins were stored at –80°C until use.

Analysis of DNA-binding activity of HIF-members family was performed through electrophoretic mobility shift assay (EMSA) as previously described (80) with some modifications. This method has been used in the study of sequence-specific DNA-binding proteins. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments containing a binding site for the protein of interest or other unrelated DNA sequences. Briefly, nuclear extracts were prepared as described above and the erythropoietin promoter hypoxia response element (HRE) oligonucleotides (Epo sense oligonucleotide 5'-GCCCTACGTGCTGTCTCACACAGC-3' and antisense oligonucleotide 5'-GCTGTGTGAGACAGCACGTA-3') were annealed in 1x annealing buffer (10mM Tris-HCl, pH7.8 and 50mM NaCl) by heating at 95°C for 5 minutes and slowly cooled down to room temperature. The double strand oligonucleotide was labelled using T4 polynucleotide kinase (Promega) after an incubation at 37°C for 30 minutes with $\gamma$-$^{32}$P[ATP]. Nuclear extracts (20 µg) were incubated with 2x binding buffer ( Hepes 40 mM, pH 7.5, NaCl 50 mM, EDTA 1 mM, DTT 1mM, PMSF 1mM, Poly(dI-dC) 1µg) for 10 minutes at room temperature. Labeled probes ($2x10^4$ cpm/µl) were added to the samples and incubated for 30 minutes at room temperature for the binding reaction. After addition of the same volume of loading buffer (Tris/HCl 250 mM, 0.2 % Bromophenol Blue, 40% Glycerol), samples were loaded onto a 5% polyacrylamide gel and run for 2 hours at 200V. In order to set the specificty of binding, a competition assay with an amount of 100, 200 and 400-fold more concentrated cold (not labeled) probe was performed with the samples expressing the maximum activity of binding (6h after treatments). Moreover, a supershift analysis of binding was observed incubating the nuclear extracts with 5µg of HIF-1$\alpha$ or HIF-2$\alpha$ antibodies 45 minutes before the binding reaction with the labeled probe.

## 2.5 IMMUNOHISTOCHEMISTRY.

Immunohistochemical analysis were performed on 4μm-thin cryostat sections fixed in methanol/acetone to localize the antigens on the hepatic tissue. After blocking non-specific binding with a solution of PBS containing 1% bovine serum albumin (Serva, Heidelberg, Germany) and 10% goat serum (Dako) for 1 h at room temperature, the primary antibodies rabbit anti-HIF-1α (Novus Biologicals) (diluted 1:100) and rabbit anti-EPAS-1 (Santa Cruz Biotech.) (diluted 1:100) were incubated overnight at 4°C on the sections. The rabbit polyclonal antibodies were detected with an Alexa-555-conjugated goat-anti-rabbit and Alexa-488 conjugated goat-anti-mouse secondary antibodies (Molecular Probes, Leiden, Netherlands). For doublestaining, each of the mentioned primary antibodies was incubated with rat anti-mouse F4/80 (Serotec) (diluted 1:100) overnight at 4°C. A mix of Alexa-555-conjugated goat-anti-rabbit and FITC-conjugated goat anti-mouse Igs (Sigma Aldrich) was successively incubated for 1h at room temperature. Sections were counter-stained with DAPI (Molecular Probes, Invitrogen) and observed with an epifluorescence microscope (Axiovert 200M, Zeiss, Germany). Negative control immunostainings were performed by omission of the primary antibody, by usage of isotype-matching control immunoglobulins.

## 2.6 *IN VITRO* ANALYSIS.

### *2.6.1 Isolation and Culture of Rat and Mouse Hepatocytes.*

Hepatocytes were isolated from male Wistar rats by circulating perfusion with collagenase I as described previously with slight modifications (79). Briefly, after anesthesia, the rat liver was perfused through the portal vein with $CO_2$-enriched calcium-deprived Krebs–Ringer buffer (120 mM NaCl, 4.83 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $KH_2PO_4$, 24 mM $NaHCO_3$) containing 0.25 mM EDTA (pH 7.4), and then digested with perfusion solution (pH 7.4) containing Krebs–Ringer buffer, 0.05% collagenase, 4 mM calcium chloride and 15 mM HEPES for 7–12 min at 37°C under recirculating conditions. The liver was excised, mechanically disrupted and rinsed in

washing buffer (pH 7.5) containing Krebs–Ringer buffer (without $NaHCO_3$), 20 mM HEPES and 0.4% BSA. Hepatocytes were then resuspended in the culture medium (M199 supplemented with 2 g/l BSA, 20 mM $NaHCO_3$, 10 mM HEPES, 1 nM insulin, 100 nM dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin), plated onto 60-mm Falcon tissue culture dishes previously treated with rat tail collagen type I at a density $2 \times 10^6$ cells per dish and incubated at 37°C in humid atmosphere containing 95% humidity and 5% $CO_2$. Fetal calf serum (5%) was present during the plating phase up to 4 h, and afterward the cells were cultured under serum-free conditions. For the *in vitro* experiments serum free medium was changed after 24 h from isolation and hepatocytes were stimulated with the acute phase cytokine, rat IL-6, IL-1β and TNF-α (PeproTech GmbH) at a single concentration (100 ng/ml medium) 1h, 3h, 6h and 12h after stimulation. A dose-dependent (1ng/ml, 10ng/ml, 100ng/ml) study was also performed at a single time point, 3h after stimulation, for all the cytokines.

On the basis of the same protocol described above, hepatocytes from C57BL/6J mouse livers were isolated by a two step enzymatic dissociation as described above, with some modifications (81). Briefly, the liver was perfused at a rate of 10 ml/min with a perfusion buffer for 5 minutes and then with a second buffer containing 0.01% collagenase Type I for 5 minutes at a rate of 12 ml/min. Hepatocytes were then dispersed, filtered and centrifuged at 200 g. The isolated cells were cultured in Dulbecco modified Eagle medium containing 10% fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamin, 10nM dexamethasone and 10nM insulin for 24h prior stimulation with cytokines. For the stimulation with IL-6 at different concentrations (1 ng/ml, 10ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml) the hepatocytes a serum free medium containing 0.2% bovine serum albumin was used.

### *2.6.2 Non-parenchymal cells isolation from rat liver.*

Rat liver macrophages (Kupffer cells) were isolated according to the method of Knook and Sleyster (82) as previously described with slight modifications. The liver was perfused with preperfusion

medium containing Gey's Balanced Salt Solution (GBSS) and sodium hydrocarbonate, followed by perfusion with enzyme solution 1 containing pronase with subsequent change to enzyme solution 2 containing pronase and collagenase. Nonparenchymal liver cells were separated using Nycodenz®(Axis-Shield PoC, Norway) density gradient as ollows: the cell suspension was transferred into four sterile 50 ml polypropylene tubes and centrifuged for 5 min at 640 g at 4°C. The supernatant was discarded and the pellets were resuspended in small volume (5-6 ml) of GBSS with 100 µl DNase I and pooled together in one sterile 50 ml polypropylene tube. 14 ml of 30% Nycodenz was added and the volume was adjusted to 24 ml with GBSS. This mixture was divided between four sterile 15 ml polypropylene tubes and GBSS (1.5 ml per tube) was carefully layered over the content of the tubes. the interphase brown layer between Nycodenz and GBSS containing nonparenchymal liver cells was transferred into sterile 50 ml polypropylene tube and washed for 5 min at 640g. The nonparenchymal liver cell pellet obtained in previous step was resuspended in 5-6 ml of 0.4% BSA/GBSS, collected in a sterile 10 ml syringe and injected in the elutriation system. Using JE-6B elutriation rotor assembled according to the manufacturer's instructions and spun at 2,500 rpm in a J2-21 centrifuge (Beckman Instruments), fractions enriched with sinusoidal endothelial cells, myofibroblasts and Kupffer cells were collected at flow rates of 19 ml/min, 23 ml/min and 55 ml/min, respectively. A particularly pure fraction (95%) of Kupffer cells was sedimented by centrifugation and cells were counted by Trypan blue staining.

### *2.6.3 Biosynthetic labelling, immunoprecipitation and NuPAGE analysis of newly synthesized erythropoietin in hepatocytes.*

Newly synthesized proteins from primary cultured mouse hepatocytes were radiolabeled as previously described (83). Briefly, mouse hepatocytes were kept in culture 24h and successively labelled in methionin-free RPMI medium supplemented with $^{35}S$ labelled-methionin (100 µCi/300µl medium pro well) for 6h. Part of the cells was further challenged with murine IL-6 at a

concentration of 100ng/ml. After the labelling, cells were wash three times in PBS whereas the supernatants were collected and diluted in 50 % lysis buffer containing 1% SDS. Cells were submitted to cycles of freezing and thawing and scraped in lysis buffer supplemented with 1% PMSF but without SDS. Cell-lysates were then harvested and diluted to 50% with lysis buffer containing sodium dodecylsulfate. The count of the total labeled proteins was measured after trichloroacetic acid precipitation of proteins. For immunoprecipitation with 5µg of rabbit anti-EPO (Santa Cruz Biotech.) in supernatants and cell-lysates, the same counts of total labelled proteins were used. Samples were pre-cleared with protein A and incubated with the antibody overnight at 4°C. Immunocomplexes were precipitated by adding 50 µl of protein A and incubation for 1 h on ice, centrifuged and washed with lysis buffer containing SDS supplemented with 0.5% BSA. Samples were finally resuspended in loading buffer containing 5% β-mercaptoethanol. Immunoprecipitated EPO was analyzed in a 4-12% Nu-PAGE Bis-Tris gel, as described above, for three hours at 80 V. Afterwards gels were fixed overnight, incubated for 1 h in AmplifyTM Fluorographic Reagent (GE Healthcare, Freiburg, Germany), covered with Cellophane and then dried. Dried gels were analyzed by autoradiography.

### *2.6.4 Immunocytochemistry.*

LabTek slides (8 wells slides) containing hepatocytes and a kupffer cells-enriched fraction after the treatment with IL-6 were washed twice with cold PBS and incubated in methanol (10 minutes)/acetone (20 seconds) for fixation. For co-localization analysis, after the blocking for 1h at room temperature as indicated in the immunohistochemistry protocol, the sections were incubated with a mixture of primary antibodies containing rabbit anti-EPAS-1 in combination with mouse anti-CK19 (Novocastra), or mouse anti-desmin (Sigma-Aldrich) or mouse anti-ED2 (Serotec), overnight at 4°C. As above, the antibodies were detected with a mixture of Alexa-555-conjugated goat-anti-rabbit and Alexa-488 conjugated goat-anti-mouse secondary antibodies (Molecular

Probes, Leiden, Netherlands). Sections were counter-stained with DAPI (Molecular Probes) and observed with an epifluorescence microscope.

## 2.7 STATISTICAL ANALYSIS

Results are shown as means ± standard deviation. The significance within groups compared to the control was established according to Student *t*-test and the intra-group analysis of variance in the cell treatment was performed with a one-way ANOVA test. Significant difference was assessed at $P < 0.05$.

# 3 RESULTS.

## 3.1 HEPATIC CHANGES OF ERYTHROPOIETIN GENE EXPRESSION IN A RAT MODEL OF ACUTE PHASE RESPONSE.

The first part of the study aimed at the identification of erythropoietin as an acute-phase protein. This concept arose from the observation of a drastic increase of serum erythropoietin concentration in the late phases of a sterile model of acute-phase reaction. Interestingly, from our studies a crucial role of the liver in the total body contribution of circulating erythropoietin under these specific conditions emerged. The new aspect illustrated in this first analysis indicates a role for erythropoietin in the patho-physiology of the acute-phase response under physiological concentration of oxygen (normoxia) possibly as a compensative mechanism in the balance of metabolic homeostasis.

### *3.1.1 Kinetics of IL-6- and IL-1-gene expression in the injured muscle after TO-injection.*

In order to confirm an effective induction of the acute phase in rats after the muscular injury, the gene expression of IL-6 and IL-1β, the two most important cytokines released by the injury site, was analyzed in the injured skeletal muscle. The number of transcripts of IL-6 increased suddenly (206 ± 40-fold increase) 30 minutes after the insult and reached a peak at 2 hours when the gene was strongly up-regulated (2900 ± 687-fold increase) (fig.1). The values gradually declined, remaining significantly higher compared to the control group up to 12h (736 ±281-fold increases). IL-1β gene expression showed a similar kinetic but the increase was about a hundred times lower compared to the IL-6 gene. In fact, the up-regulation 30 minutes after injury was 12.7 ± 7.7-fold higher and the peak after two hours represented a 95.6 ± 13.9-fold increase (fig.1).

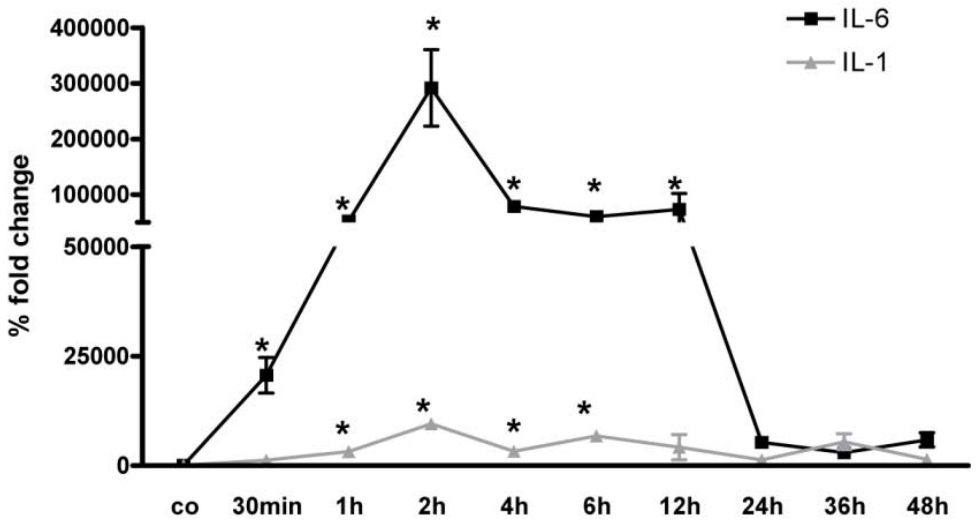



***Figure 1. IL-6 and IL-1β gene expression in the injured muscle after TO injection.*** *Analysis of the expression of the two cytokines mainly involved in the development of the acute phase reaction after local tissue injury. The relative gene expression of both cytokines was normalized using β-actin as housekeeping gene. Both the cytokines analyzed show a parallel kinetic of expression, with a significant increase already 30 minutes after the injury. They reach a peak of expression at 2 hours but the values of IL-6 gene are more than hundred-fold higher compared to the IL-1β. On the other hand, while after the peak IL-6 expression declines rapidly, the other cytokine shows a slower decrease within the kinetic. Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### *3.1.2 Serum IL-6 and EPO concentration in rats after TO injection.*

Erythropoietin serum concentration has been recently shown to increase in several clinical situations characterized by acute phase response, like heart attack, sepsis and neoplastic growth. In particular, the levels of circulating erythropoietin have been observed to correlate with the increase of IL-6 serum concentration. With this clinical foundation, a first investigation aimed at the detection of erythropoietin in rats submitted to an experimental model of acute phase reaction, intramuscular injection of turpentine oil. The concentration of IL-6 in the serum significantly increased from 2h after the injection and reached a peak of about 2.4 ng/ml at 12h after injection,

then declined steadily (fig.2a). The serum erythropoietin concentration was not detectable in control animals, as indicated by the ELISA datasheet. Circulating EPO concentration started to increase at 6h after the injury (fig.2b); at 12 hours from TO injection the EPO values in the blood reached 35 ± 15 pg/ml serum with a peak after around 24 hours (125 ± 20 pg/ml) and a gradual decrease within 48 hours. The temporal shift between hepatic mRNA expression and protein accumulation in the serum could be reasonably attributed to the time needed for processing the protein and for its release into the blood at detectable concentrations.

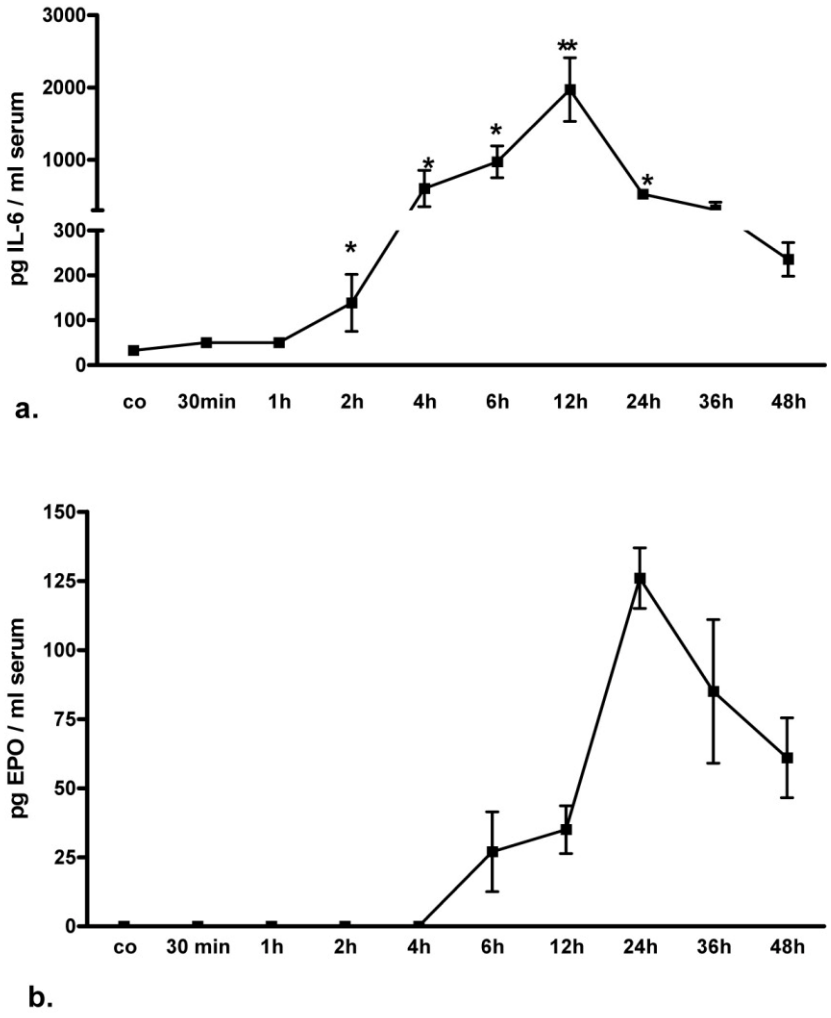



***Figure 2. Serum IL-6 and EPO levels in TO injected rats.*** *The circulating levels of erythropoietin has been found associated to acute phase conditions characterized by increased serum levels of IL-6. The graphs present an analysis of the erythropoietin serum levels in parallel to the IL-6 serum levels during acute phase reaction. The IL-6 and EPO concentration in serum samples was detected using an ELISA kit according to the manufacturer's instructions. The*

*concentration of IL-6 in the serum progressively increase starting from 2h after intramuscular turpentine oil injection to reach a peak of about 2 ng/ml at 12, afterwards gradually declines. The circulating EPO levels in control animals was not detectable as also indicated in the ELISA kit datasheet, and up until 6 hours no increase was observed after intramuscular injection (n.d.= not detectable values). The sensibility of this ELISA kit is around 8.5 pg/ml. At 6 hours an increase of EPO concentration in serum (27 ± 25 pg/ml) becomes detectable, but the values homogenously augment at 12 hours (35 ± 15 pg/ml) to reach a peak of 125 pg/ml at 24 hours, after which they decline up until 48 hours but still remain detectable. Data are expressed as mean ± S.D (N=3).*

### *3.1.3 Kinetics of EPO gene expression in the rat liver during acute-phase reaction.*

In order to individuate the possible source or sources of the glycoproteic hormone, the mRNA expression of EPO gene was investigated in all the potential organs involved in its production starting from the liver. Results obtained by Real-Time PCR analysis of total RNA indicated very low levels of erythropoietin transcripts in normal control livers as expected (low threshold cycle values from Real Time PCR analysis, 35.6 ± 0.6). The difference in expression remained insignificant compared to the control group until 2 hours after the injection. At 4 hours the values strongly increased (19.3 ± 1.7-fold increase, $P< 0.0001$), continuing to be highly expressed for 12 hours, when the absolute value showed a 19.8 ± 5.9-fold increase compared to the control (31.1 ± 0.3 PCR threshold cycle values from Real Time PCR analysis), even if with a wider individual variance ($P$=0.021) (fig. 3a). The six hours experimental group showed a slight decrease in mRNA expression (10.8 ± 2.6-fold increase, $P$=0.009) but the values remained significantly higher compared to the control group. After 12 hours gene expression gradually decreased remaining, however, significantly elevated for 36 hours.

The results of gene expression was confirmed by a semi-quantitative Southern blot analysis (fig.3b) from which it is possible to note that the band corresponding to EPO gene product (224 bp) weakly expressed in the control animal (fig.3b, lane 1) showed a progressive increase and after 12 hours remained strongly expressed (fig.3b, lanes 5 to 10).

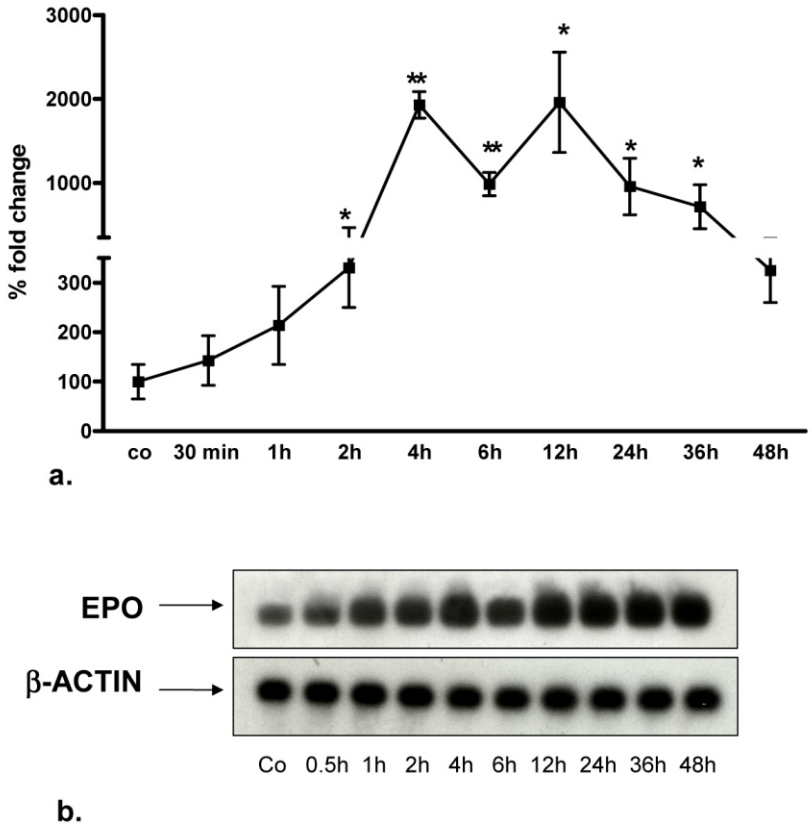
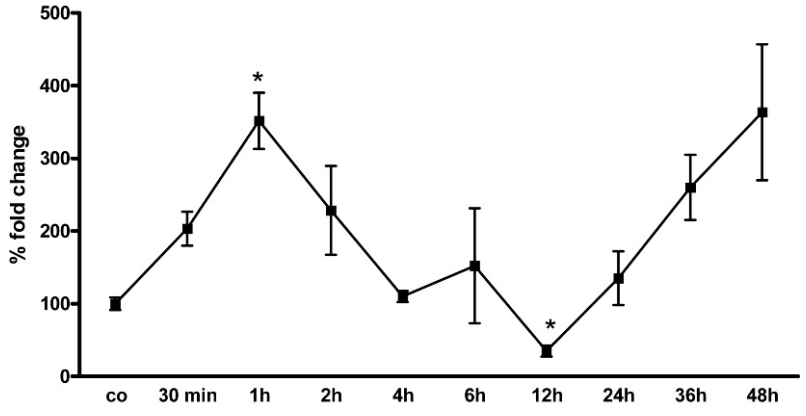


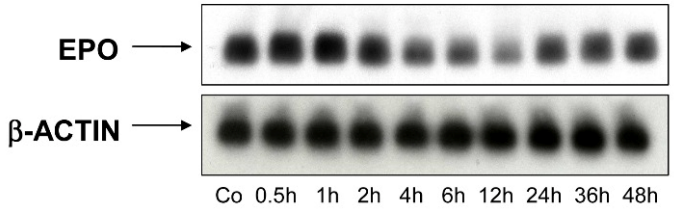

***Figure 3. EPO mRNA expression in liver of TO-treated rats analyzed by RT-PCR (a) and Southern blot analysis of PCR products (b).*** *The increase of circulating EPO is rapid and depends on de novo synthesis of the hormone which is not stored in EPO producing cells. After detecting an increase in EPO serum concentration, a further step aimed at the identification of the possible sources of the hormone. The relative expression of EPO mRNA was normalized using β-actin as housekeeping gene. The EPO gene expression significantly increases between 4 and 36 hours after intramuscular injection; at 48 hours the absolute value remains elevated although not statistically significant compared to the control (**a**). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=4). The semi-quantitative analysis of Southern blot on PCR products clearly shows the increase in gene expression and the specificity of the primers used; in figure 3 we report a representative series of animals for each time point (**b**).*

### *3.1.4 Kinetics of EPO gene expression in rat extra-hepatic organs (kidney, heart, lung and injured muscle).*

The up-regulation of erythropoietin gene in the liver detected during acute phase reaction, induced to analyze the expression also in extra-hepatic organs in order to clarify the role of the liver in this context. EPO gene expression was suddenly up-regulated in the kidney within the first hour, with an up to 3.5 ± 0.5-fold increase ($P$=0.008) (fig. 4a). The values decreased constantly afterwards leading to a significant down-regulation 12 hours after the stimulus (3.5 ± 0.9-fold decrease), the period in which the hepatic production shows its maximum up-regulation (fig.3a). The down-regulation in the kidney was confirmed by Southern blot analysis of PCR products (fig. 4b). No significant changes in EPO gene expression was recorded in heart and lung at any time after TO-administration (fig. 5a and fig. 5b) and spleen showed a similar behavior. No EPO mRNA was detectable in the injured muscle (data not shown) through PCR analysis.




a.

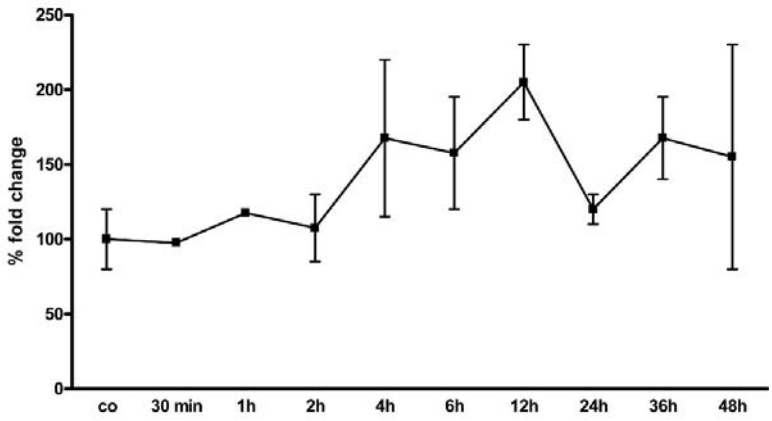



b.

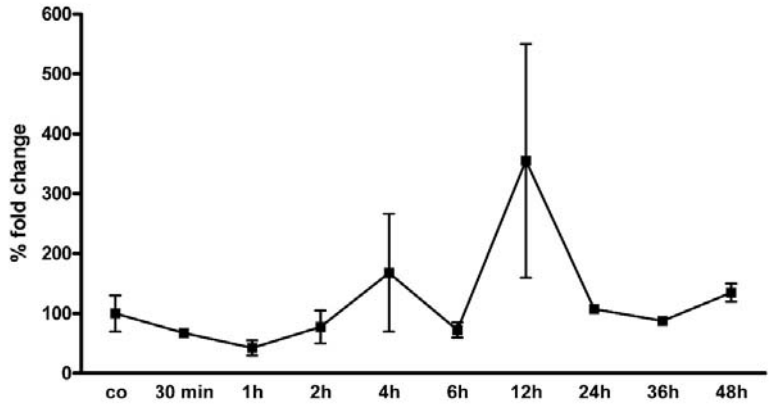
***Figure 4. EPO mRNA expression in the kidney of T.O.-treated rats analyzed by RT-PCR (a) and Southern blot analysis of PCR products (b).*** *In adult mammals, during physiologic steady state and hypoxia the kidney is supposed to be the main producer of erythropoietin. The analysis of EPO mRNA expression in the kidney during acute phase conditions revealed a progressive down-regulation up to 12h. The relative expression was normalized using β-actin as housekeeping gene. In the kidney the EPO expression reach a significant increase 1 hour after intramuscular injection, but the increase is lower compared to that seen in the liver. The expression declines during the 12 hours with a progressive down-regulation significantly lower than the control group (**a**). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, analyzed by Student's t-test, n=4). The semi-quantitvative analysis of Southern blot on PCR products better illustrates a reduction of the intensity of expression of a representative series of animals for each time point (**b**).*

### *3.1.5 Hepatic tissue protein concentration after TO injection.*

In total liver homogenates (fig. 6a), an increase of the tissue EPO concentration compared to the control group (8.3 ± 0.5 pg/mg tissue protein) was detected 4 hours after the TO injection (13.1 ± 2.2 pg/mg tissue protein respectively). Western blot analysis showed a progressive weak increase




a.




b.

***Figure 5. EPO mRNA expression in heart (a) and lungs (b) of T.O.-treated rats analyzed by RT-PCR.*** *Minimal amount of EPO mRNA has been detected also in extra-renal organs other than the liver. In order to consider other possible sources of the circulating hormone a RT-PCR analysis was also performed on RNA extracted from organs such as heart, lungs and spleen. The relative expression was normalized using β-actin as housekeeping gene. No significant increase compared to the control group was detected, in spite of an increase in absolute value at 12 hours in the heart (**a**) and in the lungs (**b**). Results are expressed as mean ± S.E. (P<0.05, analyzed by Student's t-test, n=4).*

of the protein content in the liver which became clearly evident after 6 hours from the injection, and was related to the increase observed in the serum levels (fig. 6b). It is important to underline that very low amounts of EPO are stored in the cells and after its synthesis, the protein is quickly processed and released into circulation; this could explain the low presence in the tissue, while at

the same time a prominent and gradual increase of the circulating levels in parallel with the gene up-regulation.

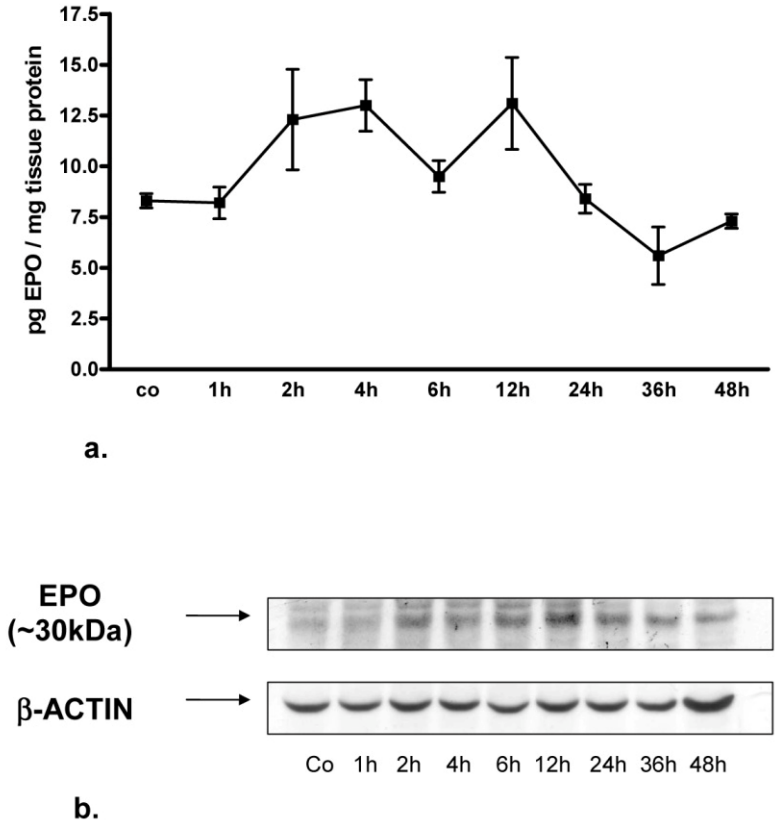



***Figure 6. Quantitative analysis of EPO tissue protein expression in liver tissue by ELISA assay (a) and western blot analysis (b).*** *In order to obtain a quantitative analysis of EPO protein expression, liver homogenates of two series of animals for every time point were processed by ELISA assay. Control groups showed the lowest amount detectable with the assay (around 8.2 ± 0.5 pg/mg tissue protein). In the hepatic total homogenates (**a**), a significant increase was observed at 4 and 12 hours (13.0 ± 0.5 pg/mg tissue protein and 12.8 ± 2.0 pg/mg tissue protein). In the western analysis (**b**), the band corresponding to EPO molecular weight (signal detected around 30 kDa) is weakly present in normal liver, as also weakly expressed at mRNA level, and the band intensity is lightly increased after four hours and constantly expressed up until 48 hours, confirming the kinetic of expression observed for the mRNA. The low levels of tissue erythropoietin content might be explained by the low amount of protein stored in the cells and by a rapid release of the protein into the bloodstream.*

### *3.1.6 Turpentine oil injection induces an up-regulation of the HIF-1α gene expression but an increase of the protein levels of both HIF-1α and HIF-2α.*

Along with the expression of EPO, a further study aimed at the identification of a possible correlation between erythropoietin mRNA increase and the expression of HIF-1α and HIF-2α, the transcription factors mainly involved in the regulation of the glycoprotein hormone. Our studies showed an up-regulation in HIF-1α-gene expression starting from the second hour after injection and reaching a maximum of expression between four and six hours. The increase was 4- to 5-folds compared to the control animals, indicating an up-regulation of the gene coding for a protein that in physiological normoxic conditions is subject to hydroxylation, ubiquitination and to proteosomic degradation. These post-translational modifications could explain the differences in the pattern of temporal expression between mRNA and protein. In these experimental conditions, no significant variations in the expression of HIF-2 alpha gene were detected (fig 7a).

The analysis of HIF-1α protein levels detected in total hepatic homogenates showed a progressive increase of the signal up until 24h after the injection, confirming a reduced degradation and an increased stabilization of the protein. In the same total protein extracts the increase of HIF-2α expression reached its maximum between 4h and 24h hours after the insult (fig.7b).

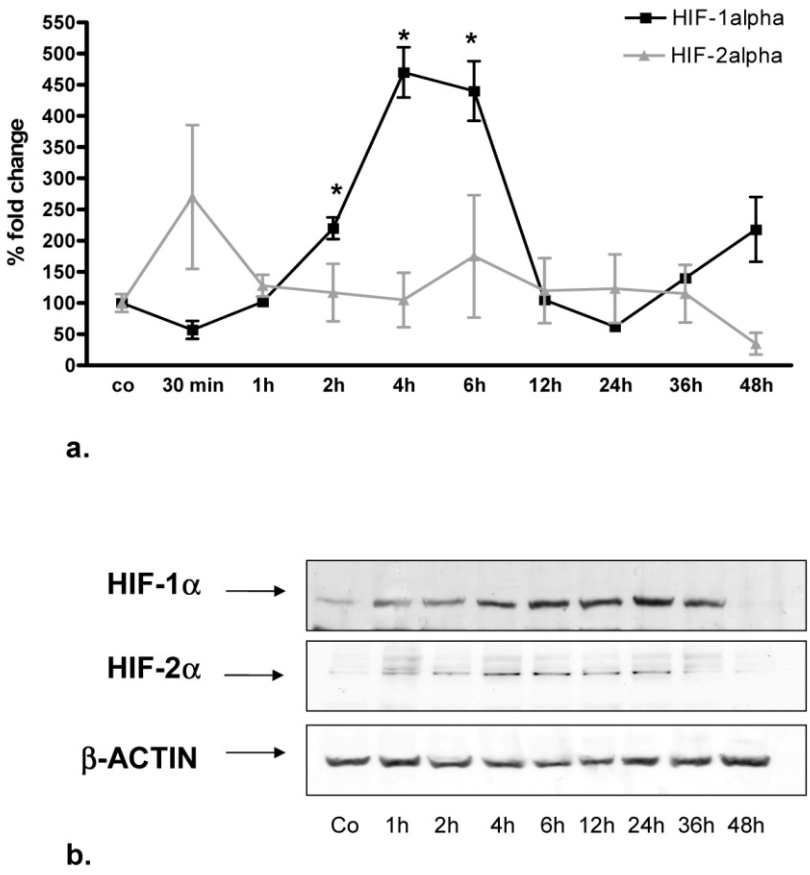



***Figure 7. HIF-1 alpha and HIF-2 alpha mRNA and protein expression in liver of TO-treated rats analyzed by RT-PCR (a) western blot (b)****. Extensive studies have been focused on erythropoietin gene expression induced by hypoxia via the hypoxia inducible factor (HIF) family of transcription factors. A genomic and proteomic evaluation for HIF-1α and HIF-2α was performed in order to establish a possible correlation with erythropoietin gene expression. The relative expression of HIF-1 alpha gene was normalized using β-actin as housekeeping gene. HIF-1 alpha expression significantly increases between 4 and 6 hours (4.7 ± 1.7-fold increase, P=0.045, and 4.4- ± 0.8-fold increase respectively, P=0.0051) after the TO injection. No significant changes in HIF-2 alpha gene expression were detected. (**a**). Data are expressed as mean ± S.D. * Values statistically significant compared to the control group (P<0.05 , analyzed by Student's t-test, N=4). Western blot analysis on total protein extracts (40µg) **(b)** indicates a gradual increase of accumulation of the HIF-1α protein up to 24h after treatment and a similar increment of HIF-2α levels between 4h and 24h clearly emerges from this representative series of samples. Post-transcriptional modifications influencing the stability of HIF members might justify the different behavior observed among gene and protein expression.*

### *3.1.7 Acute phase cytokines-induced changes of EPO gene expression in isolated hepatocytes and Kupffer cells.*

In order to confirm a possible direct effect of the pro-inflammatory cytokines on EPO gene induction, we stimulated primary cultures of rat hepatocytes with the recombinant IL-6 and IL-1β. As shown in figure 8a, high IL-6 concentrations were able to induce an effective increase (about 4-fold increase) of EPO-specific transcripts at 3 hours after stimulation, and stimulation with IL-1β induced a light up-regulation (about 2-fold increase) in a dose-independent manner. Low doses of TNF-alpha did not alter EPO gene expression whereas high concentrations induced a down-regulation observed also in the kinetic analysis. Analyzing the kinetics (fig.8b), RT-PCR revealed an early induction of the EPO gene expression 1 hour after the stimulation (around 2.5-fold increase). The mentioned acute phase cytokines were able to induce a rapid up-regulation of the gene with a further increase at 3 hours (5.6 ± 0.9-fold increase). The values remained significantly higher up until 6 hours and gradually declined within 12 hours. Interestingly, the stimulation with IL-6 and IL-1β was also able to up-regulate HIF-1 alpha gene expression in hepatocytes with a similar kinetic to the one observed for EPO gene (data not shown).

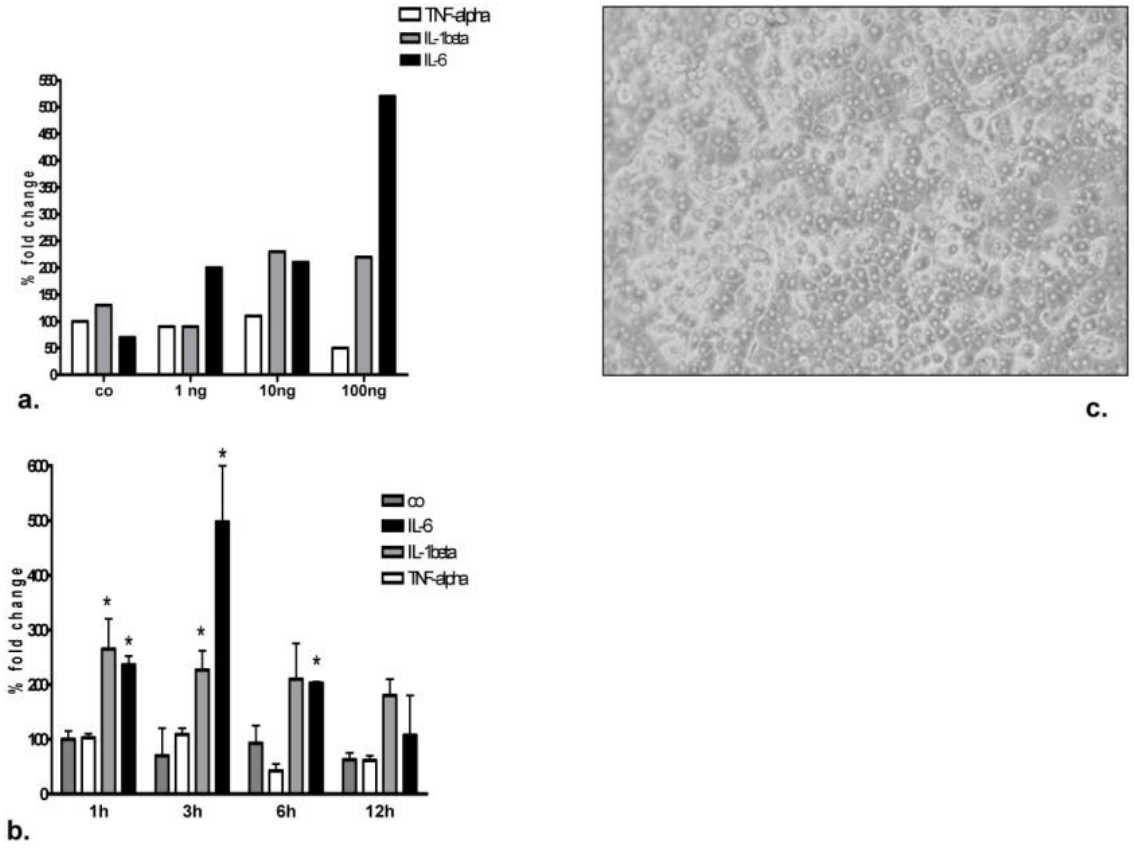



***Figure 8. EPO gene expression in isolated rat hepatocytes after stimulation with acute phase cytokines through Real-Time PCR.*** *The hepatocyte represents the main cell population of the liver involved in the acute phase protein synthesis. The acute phase cytokines treatment has been shown to influence in isolated hepatocytes the expression of the most important acute phase proteins. In order to evaluate the direct effect of these mediators on erythropoietin gene expression a stimulation in a dose- and time-dependent manner was performed on isolated rat hepatocytes. The relative expression of EPO gene was normalized using β-actin as housekeeping gene. A dose-dependent analysis reveals that high concentrations of IL-6 are able to up-regulate the EPO gene 3h after the treatment up to a 5.2-fold increase. IL-1β induced an increase in EPO gene expression of 2.5 at a concentration of 10 and 100 ng/ml, whereas TNF-α stimulation triggered a dose-dependent down regulation of the gene* ***(a)****. The stimulation with 100 ng/ml of IL-6 induced a sudden and temporarily significant increase of EPO expression within 1 hour, and at 3 hours the expression further increased to reach a peak of 5.6 above baseline. A similar kinetic was observed with the same dose of IL-1β where the values increased up to 2-fold between 1h and 3h after stimulation, whereas a progressive down-regulation of EPO was recorded after TNF-α treatment. At 6 hours the values progressively declined, and remained significantly higher*

*compared to the control group only in the IL-6 treated hepatocytes.* ***(b)*** *(*P < 0.05). Hepatocytes primary culture 24h after isolation* ***(c)*** *(Magnification 200x).*

In a similar way, considering the heterogenic hepatic cell population, an analysis of cytokine stimulation also in isolated Kupffer cells, resident macrophages in the liver, was performed. Although hepatocytes and fibroblasts-like cells are supposed to represent the major sources of erythropoietin in the liver, the production of the hormone has been also demonstrated to occur in activated macrophages (84). Interestingly, the main cytokine able to produce an erythropoietin up-regulation was IL-6, as reported in figure 9. Indeed, stimulation with other acute phase cytokines (IL-1β and TNF-α) or following LPS treatment did not result in an induction of erythropoietin gene (data not shown).

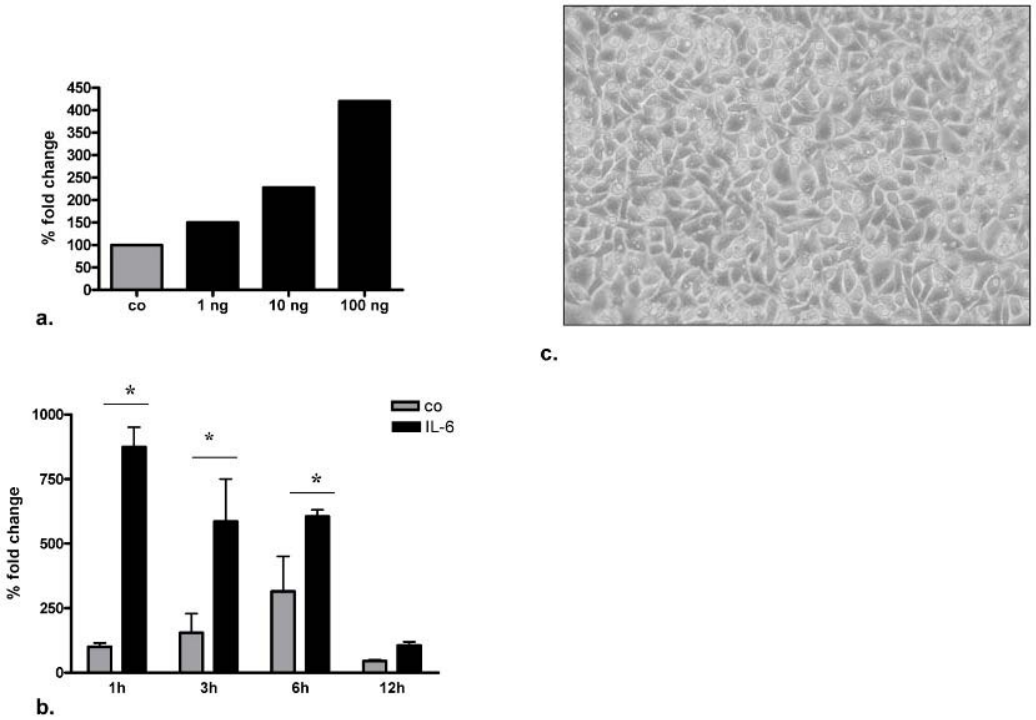



***Figure 9. EPO gene expression in isolated rat Kupffer cells after stimulation with the acute phase cytokine IL-6 through Real-Time PCR.*** *The liver is an organ composed by a wide heterogeneous cell population. Kupffer cells, resident macrophages of the liver, has been previously shown to contribute to erythropoietin production under specific*

*pathological conditions. An analysis of the effects of IL-6 on erythropoietin gene expression was also considered in ex vivo isolated Kupffer cells. The relative expression of EPO gene was normalized using β-actin as housekeeping gene. A dose-dependent analysis reveals an apparent dose-dependent increase of erythropoietin gene expression following IL-6 stimulation (**a**). A kinetic analysis after IL-6 stimulation shows a sudden up-regulation of gene induction up to 8-fold already at 1h after the treatment. The values progressively decline to control levels within 12h, although persisting significantly higher compared to the control group (more than 2-fold) until 6h (**b**) (*P < 0.05). Kupffer cell primary culture 24h after the isolation (**c**) (Magnification 200x).*

## 3.2 IL-6 PLAYS A KEY ROLE IN THE HEPATIC REGULATION OF ERYTHROPOIETIN GENE EXPRESSION IN VIVO AND IN VITRO.

From the results obtained in the first study, an important role for IL-6 in the induction of erythropoietin during acute-phase reaction emerged. In order to understand the role of IL-6 in Epo-gene up-regulation *in vivo*, a second series of experiments was performed with the use of IL6-knockout mice and with a parallel analysis of a further model of acute-phase response. The importance of a comparison between a sterile (TO) and a septic (LPS) model is mainly justified by the influence of different mediators and cell populations orchestrating the development and the resolution of the acute-phase. Clinically and biologically, each individual responds differently to different patho-physiological conditions and several mechanisms of compensation are triggered upon the influence of the biological microenvironment. The regulation of the balance among these mechanisms might result in an exacerbation or a recovery of the process. Moreover, another important question concerned the expression of HIF-1α and HIF-2α in absence of IL-6 and their possible correlation with erythropoietin-gene induction. Interestingly, the localization of these two transcription factors appears to be cell-specific in the hepatic parenchyma: hepatocytes together with macrophages express mainly HIF-1α during acute phase, whether other extra-parenchymal cell populations (endothelial cells and fibroblasts) seem to express predominantly HIF-2α. The aim of

this second study was to evaluate the effects of a lack of IL-6 on erythropoietin production and on the main transcription factors known to regulate its expression.

### *3.2.1 Differences in the pattern of acute-phase cytokines and serum amyloid A-1 between wild type and IL6-knockout mice.*

The use of an IL6-knockout mice strain in order to analyze possible changes in the hepatic erythropoietin gene expression imposed the use of a respective wild type strain as a control (C57BL/6). On the basis of previous results from the rat experimental model, the comparison between species and between different strains resulted mandatory in order to understand changes in the development of the acute-phase response that could potentially affect the erythropoietin regulation.

#### **3.2.1.1. Acute phase response cytokines gene expression in mice after intra-muscular turpentine oil injection.**

The acute phase response induced by turpentine-oil injection is characterized mainly by a massive systemic release of IL-6 and a local production of IL-1β from the injured muscle, as previously reported. As illustrated in fig.10a, the values of IL-6 and IL-1β progressively increased already from the second hour after the injection to peak at 6h with an expression of 297±74-fold increase and 135±19-fold increase respectively. The serum concentration of IL-6 gradually augmented until 12h after the injury with values of about 40 ng/ml (data not shown). The expression of TNF-α never reached values statistically different from the control group during the analyzed kinetic. In the IL-6 knockout animals the lack of IL-6 appeared to be compensated by a stronger induced augment of IL-1β which reaches a peak of 450-fold increase at 12h after the injection (fig.10b). In this group also TNFα gene resulted significantly up-regulated with a maximum of expression up to 115±45-fold increases at 12h.

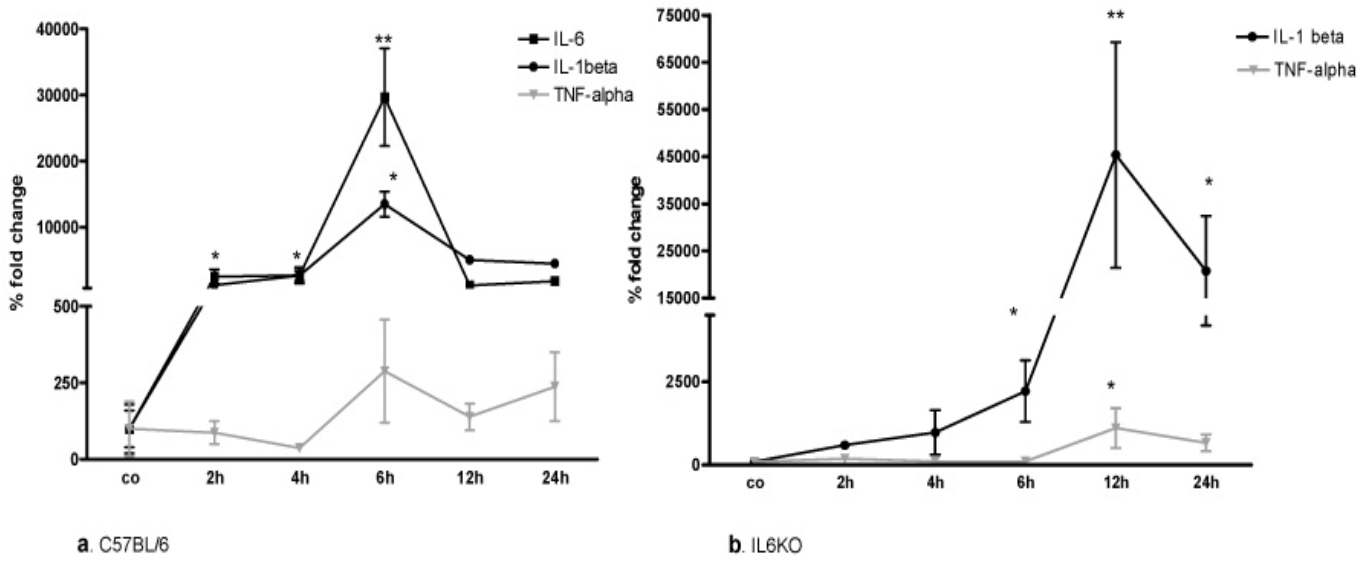



***Figure 10. Pattern of acute phase cytokines expression in TO injured muscles of wild type (a) and IL6KO mice (b).*** *Depending on the nature of the stimulus inducing the tissue damage, different patterns of cytokines and mediators contribute to the development of the acute phase response. The lack of a particular mediator or cytokine might induce the activation of compensative mechanisms able to alter the organism response. The relative expression of each cytokine was normalized using β-actin as housekeeping gene. In the wild type strain IL-6 and IL-1β show a parallel kinetic of expression, with a significant up-regulation already at 2h after injection (**a**). They reach a peak of expression of 300-fold increase and 130-fold increase respectively at 6h after the injection followed by a progressive decline. No significant change in TNF-α gene expression is observed. In the IL6KO strain the absence of IL-6 seems to be compensated by a stronger delayed up-regulation of IL-1β up to 450-fold increase at 12h after the injury with a similar increment of TNF-α up to 110-fold increase (**b**). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### 3.2.1.2. Acute phase response cytokines gene expression in mice after intra-peritoneal LPS administration.

The acute phase response induced by LPS administration is known to be triggered mainly by Kupffer cells which come in contact with the bacterial component through the TLR4 receptor.

Activated Kupffer cells represent the major source of acute phase cytokines that are released in a paracrine way to stimulate the surrounding parenchymal cells and in an endocrine way via bloodstream. The cytokines pattern that characterizes the septic acute response is more homogeneous and IL-1β and TNFα represent the main inflammatory mediators released by the liver (fig.11). In the wild type group, IL-1β reaches a maximum peak at 4h up to 108±26-fold increase, whereas TNF-α after a sudden and progressive up-regulation peaks at 6h up to 58±15-fold increase (fig.11a). However, IL-6 shows a sudden augment at 2h (53±6-fold increase) and remains constantly express until 6h afterward progressively declines (fig.11a). As shown in figure 2b, the lack of IL-6 in this situation seems not to alter the cytokines expression except for an earlier up-regulation of IL-1β and TNFα.

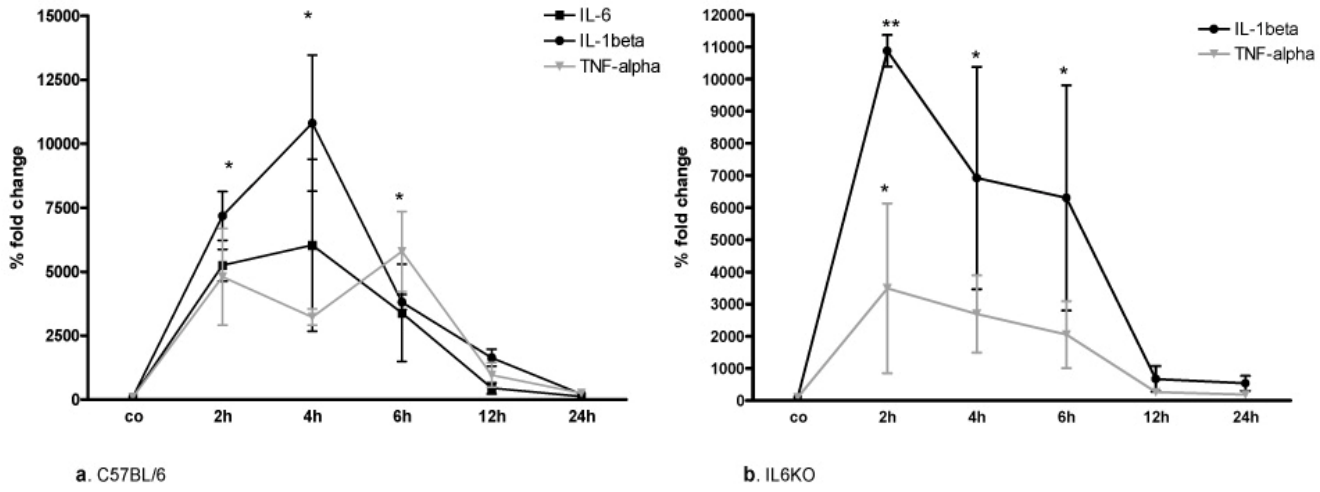



***Figure 11. Pattern of acute phase cytokines expression in the liver of LPS injected wild type (a) and IL6KO mice (b).*** *After intraperitoneal LPS administration, activated Kupffer cells in the liver produce large amount of acute phase cytokines, in particular TNF-α and IL-1β. As previously described, the IL-6 plays only a limited role in the evolution of the acute phase. The relative expression was normalized using β-actin as housekeeping gene. In this model, the induction of IL-1β expression (110-fold increase) overcomes the up-regulation of IL-6 (55-fold increase). The expression of TNF-α in this case is also significantly induced until 6h (up to 60-fold increase)(**a**). In the IL6KO strain no significant quantitative alteration are observed, but an earlier up-regulation of IL-1β and TNFα with peak of*

*expression at 2h (**b**). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### 3.1.2.3. Hepatic Serum Amyloid A-2 (SAA-2) gene expression resulted quantitatively and temporally altered in IL6-knockout mice submitted to TO model, whereas only minimal differences where detected in LPS treated animals

In order to monitor the effects of IL6 deficiency on the production of acute-phase proteins, a Northern analysis of SAA-2-gene expression, one of the most representative APR, was performed in the liver of both strains during both experimental models. A very low expression of SAA-2 gene was detectable in normal livers, whereas during acute phase reaction a strong increase of the signal was already evident at 2h and progressively augmented along all the studied kinetic after TO-injection and peaking at 12h after LPS administration in the wild type animals (fig.12a). In the IL6KO strain, the serum amyloid A-2 gene expression appeared almost unaltered in the LPS group, whereas the expression showed an anticipated increase compared to the wild type animals in the TO-treated group with a progressive reduction after 6h (fig.12b). The differences in the temporal pattern of expression between the two strains indicate an important role of IL-6 in the evolution of acute-phase response particularly in the TO model, as previously shown. On the other hand, the induction of acute-phase proteins appeared slightly reduced in the LPS group indicating compensatory mechanisms capable to sustain an efficient response of the organism to the injury.

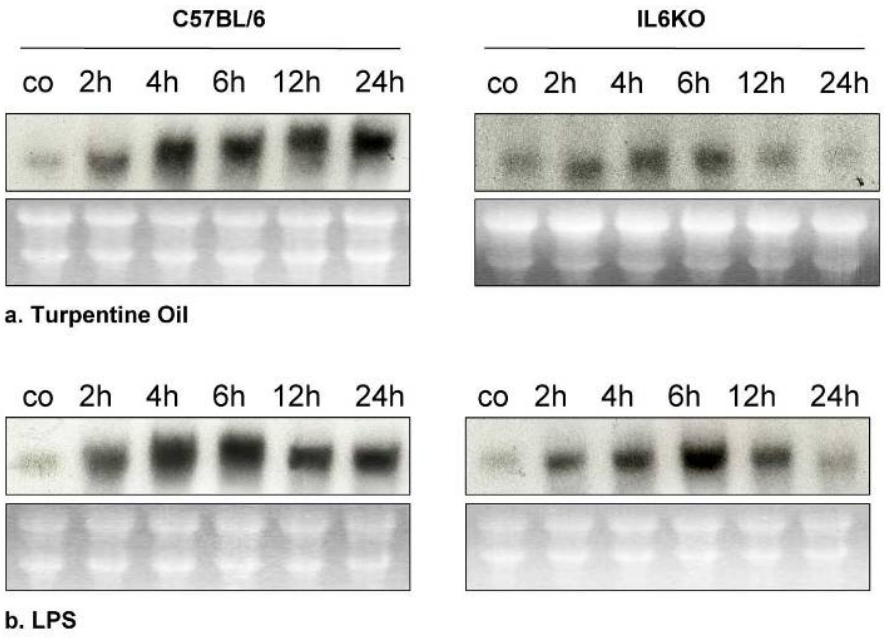



***Figure 12. SAA-2 hepatic gene-expression during acute-phase reaction.*** *The synthesis of acute phase proteins in hepatocytes is critically influenced by the pattern of cytokines triggered by the tissue damaging agent. Although several mechanisms of compensation exist in nature, the balance of cytokine produced might positively or negatively regulates the synthesis of certain acute phase proteins. A comparison of SAA-2 expression between two models of acute phase resulted necessary to establish the importance of different mediators on the acute phase proteins synthesis.Moreover, the analysis of SAA-2 gene expression was performed to show an effective induction of acute phase conditions in spite of changes in the pattern of cytokines expression. The Northern blot on total RNA extracted from mouse liver submitted to two models of acute phase reaction was used as a semi-quantitative analysis of gene expression. In both models the lack of IL-6 modestly affects the expression of the acute-phase protein. In particular, IL-6 knockout animals submitted to turpentine injection (**a**) present a lower up-regulation of the gene that rapidly decreases. In LPS treated mice (**b**) the induction of SAA-2 results minimally influenced by IL-6 deficiency.*

### *3.2.2 Differences in the erythropoietin gene-expression pattern between two different mice strains submitted to two different experimental models.*

Once the gene expression of the main acute phase mediators involved in both acute phase models in the different mice strains was recorded, the serum erythropoietin concentration and the hepatic

expression of the gene were analyzed in order to monitor *in vivo* the effects of different acute phase conditions influencing the production of the hormone particularly with a deficit of IL6.

### 3.2.2.1. EPO serum concentrations after acute-phase response induction in mice.

EPO serum concentration resulted increased of about 2-fold compared to control animals already 2h after TO injection slightly declining to the control levels within 6h. At 12h we recorded a sudden increase that reached 10-fold changes to gradually decline at 24h after the insult (fig.13a). In the IL6O strain after a light initial increase of about two fold, the levels of circulating EPO progressively decline until 24h (fig.13a). The sudden increase at 2h followed by a modest decline was also observed in the LPS treated mice and a similar peak was registered at 12h, but the increase was only 3.5 fold compared to the normal animals (fig.13b). In the IL-6KO mice the serum EPO concentration did not overcome 2 fold increases in every time points analyzed (fig.13b).

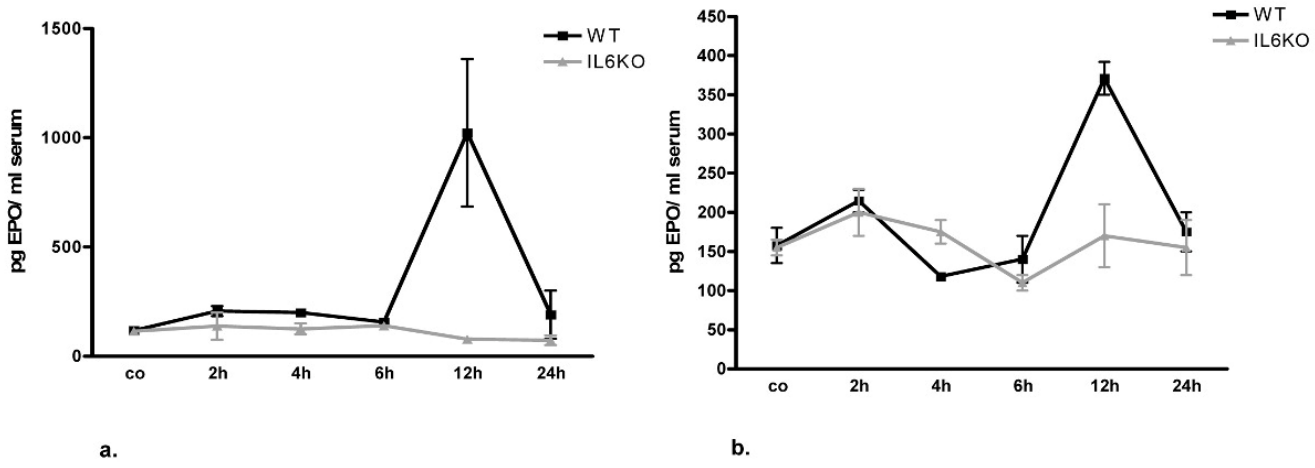



***Figure 13. Serum EPO concentration in TO (a) and LPS (b) treated animals.*** *In order to monitor the circulating levels of erythropoietin during two different models of acute phase response, the EPO concentration in serum samples was detected using ELISA kit according to the manufacturer instructions. The serum concentration of EPO in normal mice is around 110 pg/ml. After a sudden 2-fold increase, a progressive decrease up to the control levels is recorded*

*until 6h after the treatments in wild type animals (**a**). At 12h the circulating levels of EPO reach a concentration of almost 2 ng/ml in the TO injected group and 350 pg/ml in the LPS treated group. In the IL6KO strain the concentration of EPO progressively declines after the muscular injury and does not increase over 2-fold in the LPS stimulated animals (**b**). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### 3.2.2.2. Acute phase reaction in both experimental conditions results in an up-regulation of EPO gene expression in the liver of wild type mice, but no significant increase in the liver of IL6KO mice.

A parallel comparison of erythropoietin gene expression in the liver between two different models of acute phase was successively performed. The hepatic values of EPO gene expression increased significantly in both models of acute phase response in wild type mice. Even if with different kinetics of expression, the values augment progressively to reach a peak of 8.2 ± 2.2-fold increase at 12h in the TO-injected animals (fig.14a), whereas in the LPS-treated the peak of expression of 4.6±1.1-fold increase was recorded at 6h followed by a progressive decline until 24h (fig.14b). In the IL6KO animals the increase of EPO gene expression resulted dramatically reduced to 1.8±0.1-fold increase at 6h in the TO model and a similar drastic decrease to 1.5-fold increase was observed in the LPS-treated animals at 6h (fig. 14a and fig.14b respectively). The western analysis (fig.16a) showed a similar progressive increase of the EPO protein hepatic until 12h after TO-injection and until 4h in the LPS-treated wild type mice. Both the treatments induced a weaker protein increase in the liver of IL6KO mice (fig.16b). The densitometric analyses clarified the increase of the protein expression showing a peak at 12h and 6h respectively for turpentine oil and LPS treated animals. Of note, EPO is a glycolproteic hormone stored in the cell in very low amounts and the newly synthesized protein is supposed to be quickly secreted in the circulation. This could explain the low increase in the tissue protein content compared to the high messenger RNA induction.

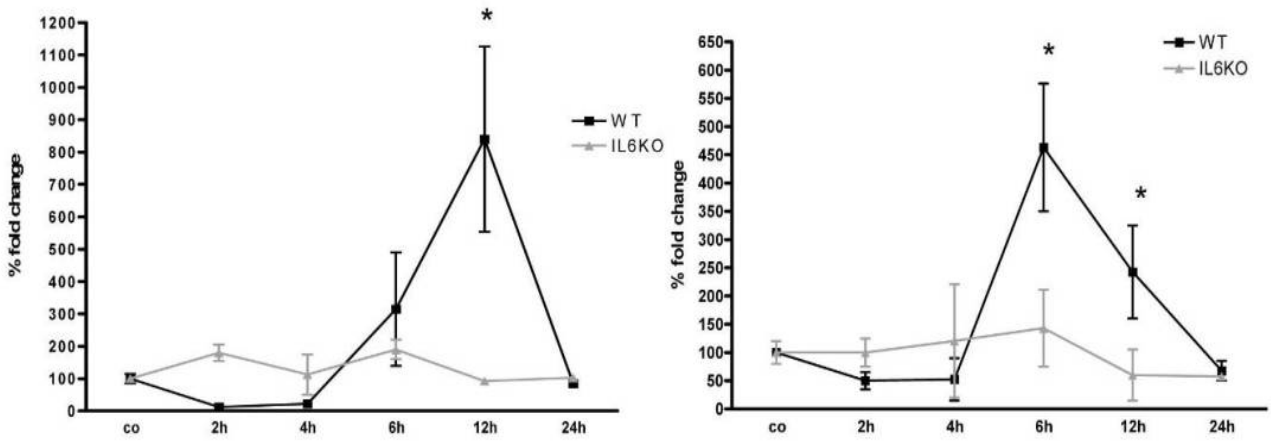



***Figure 14. EPO mRNA hepatic expression of wild type and IL6KO after TO injection (a) and LPS (b) treatment.*** *After the detection of erythropoietin in the serum, a successive step aimed at the evaluation of erythropoietin gene expression in the liver of both mice strains. The relative expression was normalized using β-actin as housekeeping gene. EPO gene expression results significantly up-regulated at 12h after TO injection in the wild type strain (8.2±2.2-fold increase) after an initial decrease, whereas in the IL6KO strain the increase reaches 2.0±0.4-fold increase at 6h and progressively declines to control level within 12h (a). LPS treatment induces a lower increase between 6h and 12h after the injection in the wild type group (4.6±1.1-fold and 2.5±0.8-fold increase respectively). EPO gene expression remains almost unvaried in the IL6KO mice treated with LPS (b). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

Interestingly in mice, after TO-injection the kidney reflects a similar kinetic of expression observed for the liver (data not shown), but with a lower up-regulation up to 2.5-fold increase at 12h in terms of mRNA expression. The LPS treatment induced a similar up-regulation of EPO mRNA expression both in liver and kidney with a similar temporal pattern of induction. Lack of IL-6

almost totally reversed the renal EPO mRNA increase in both experimental models (data not shown).

### *3.2.3 Analysis of expression of the main erythropoietin transcription regulators, HIF-1α and HIF-2α, in the IL6-knockout strain during acute phase reaction.*

**3.2.3.1. Acute-phase reaction induced in both the experimental conditions results in an up-regulation of HIF-1alpha gene and protein expression in the liver of wild type mice, but a significantly lower increase in the liver of IL6KO mice.**

The RT-PCR analysis (fig.15) shows a parallel progressive up-regulation of HIF-1α gene common to both the experimental models of acute phase response. The degree of the augment in the number of transcripts is similar in TO- and LPS-treated wild type mice (maximum 5.0±1.5-fold increase and 5.9±1.4-fold increase respectively at 6h) (fig.15a-b). In the IL6KO strain no significant increase in the number of transcripts is observed after the TO administration, whereas in the LPS-treated group HIF-1α gene up-regulation reaches a peak of 3.7±1.5-fold increase at 6h.

The western analysis (fig.16a) shows an augment of the signal already at 2h, but particularly evident at 12h after TO-treatment in wild type mice, whereas in the LPS treated animals a stronger expression is registered within 6h from the injection. In the IL6KO animals (fig.16b) the relative augment in the protein content was less prominent in both the acute phase reaction models. The analysis of binding performed by electrophoretic mobility shift assay revealed a progressive increase of HIF activity after TO-injection with a particular strong intensity of binding between 4h and 6h (fig.17a-b). A similar situation can be observed after LPS treatment where the radiography shows an earlier increment of the signal at 4h (fig.17a-b). The competition assay and the supershift analysis indicate that a part of the binding protein is constituted by HIF-1α (fig.17c).

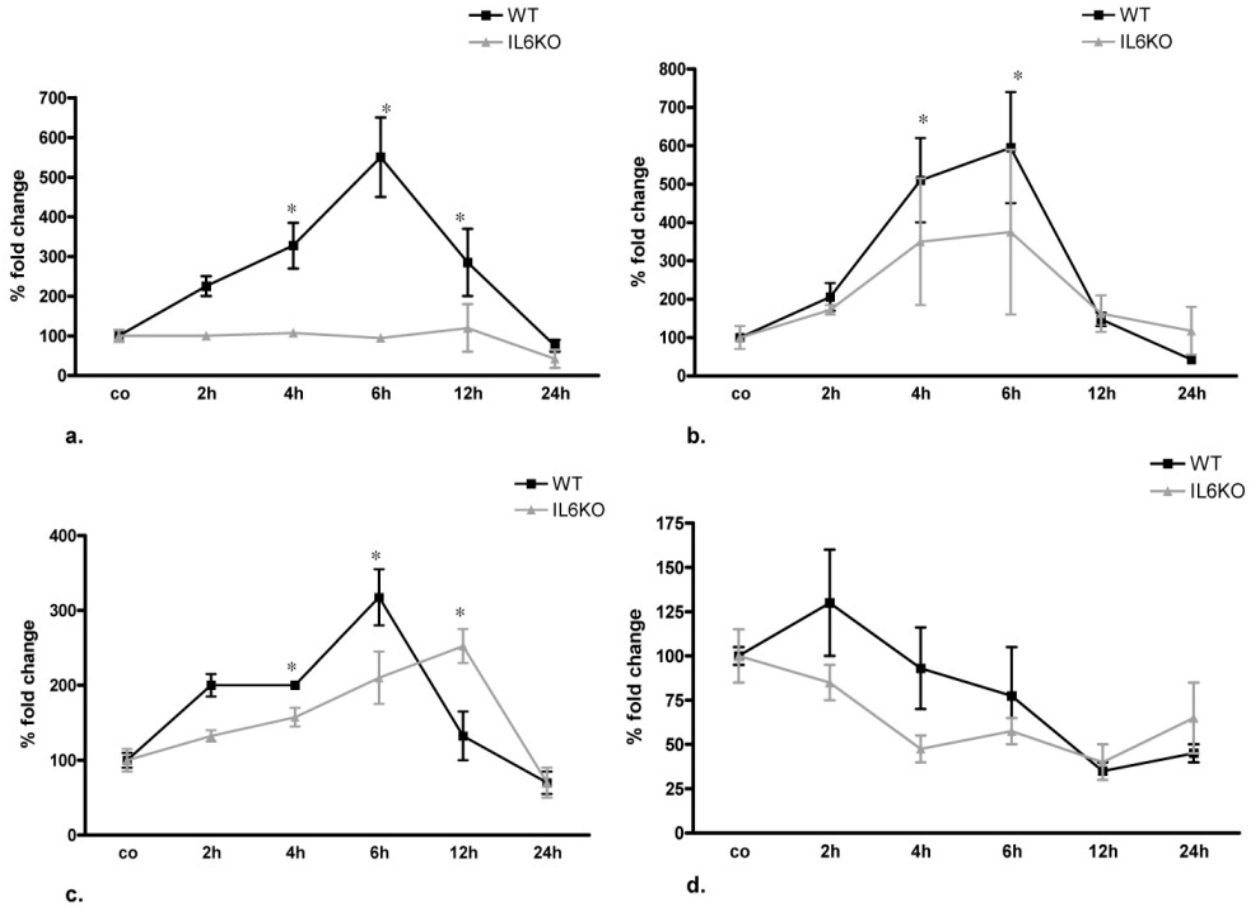
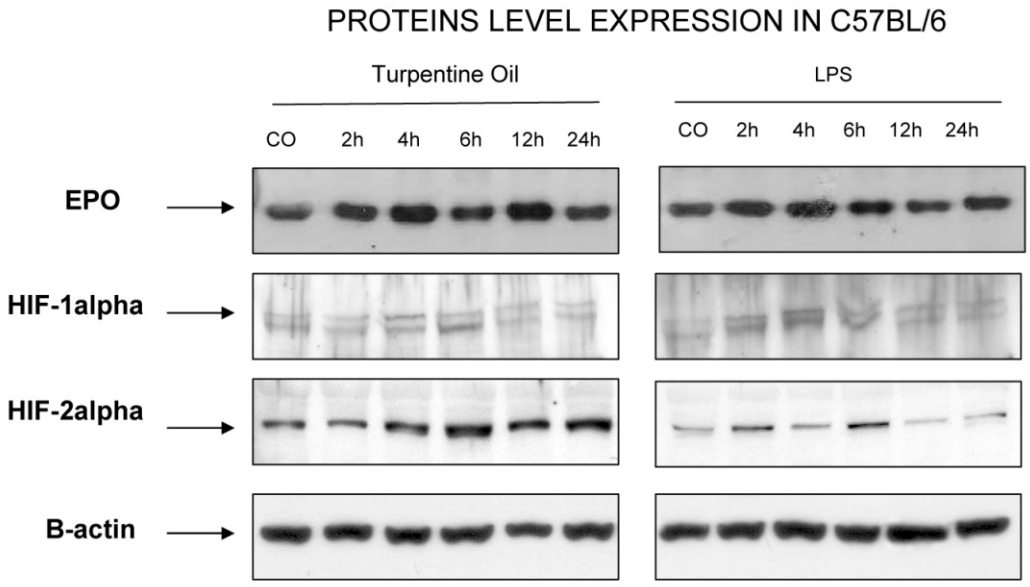



***Figure 15. HIF-1α (a-b) and HIF-2α (c-d) mRNA hepatic expression of wild type and IL6KO mice after TO injection (a-c) and LPS (b-d) treatment.*** *Although mRNA levels and protein expression of these transcription factors might be differentially regulated, an up-regulation of gene expression could contribute to an increase of protein levels with a consequent translocation of the protein in the nucleus. Furthermore, an analysis of gene expression was performed to consider which cytokines might influence HIF-1α and HIF-2α gene expression. The relative gene expression of both transcription factors was normalized using β-actin as housekeeping gene. HIF-1α gene expression follows a progressive increase up to 5.0-fold increase at 6h hours after TO injection in the wild type animals (**a**). Similarly, after LPS stimulation the hepatic changes of HIF-1α peak up to 5.9-fold at 6h (**b**). The lack of IL6 in vivo resulted in a dramatic inhibition of HIF-1α gene up-regulation after acute phase response, particularly evident in the TO model (**a-b**). HIF-2α gene expression results significantly augmented after TO injection in the wild type group, with a delay in the kinetic in the IL6KO strain (3.2±0.4-fold and 2.5±0.1-fold increase respectively) (**c**). LPS treatment induced a progressive down-regulation of HIF-2α gene expression up to 12h particular evident in IL6KO mice (**d**).*

*Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### 3.2.3.2. HIF-2alpha gene expression increases after TO treatment in both the mice strains, but LPS injection induces only an augment of the protein levels without affecting the expression.

The HIF-2α mRNA levels are particularly high in the normal liver, but the protein content results in a minimal detectable concentration. Only after TO-injection an up-regulation of HIF-2α gene expression with a peak of 3.2±0.4-fold increase at 6h for the wild type and a peak of 2.5±0.2-fold increase at 12h in the IL6KO mice (fig.15c-d). Interestingly both the models of acute phase were able to induce a stabilization of the protein in wild type and IL6KO mice, as reported in the western analysis (fig.16a-b). This result clearly indicates that, for HIF-2α, the gene expression and the protein stabilization are independently regulated. Moreover the radiography obtained after supershift analysis in figure 17c shows an almost complete disappearance of the signal after incubation with HIF-2α antibody indicating a further involvement of this member in the transcription complex.


PROTEINS LEVEL EXPRESSION IN C57BL/6
Turpentine Oil
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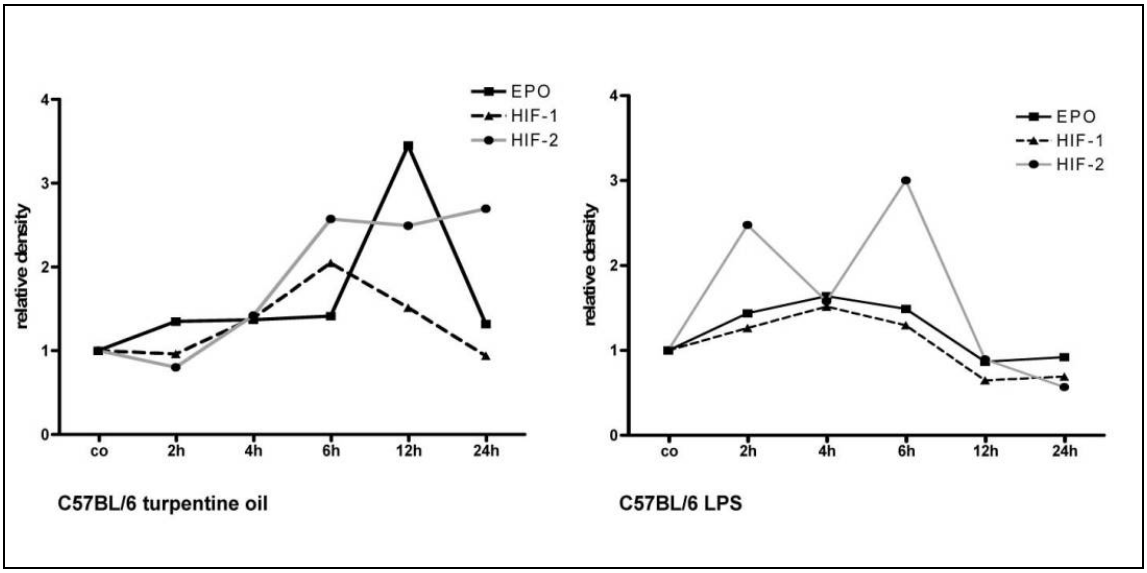
CO 2h 4h 6h 12h 24h
EPO
HIF-1alpha
HIF-2alpha
B-actin

**a.**


EPO
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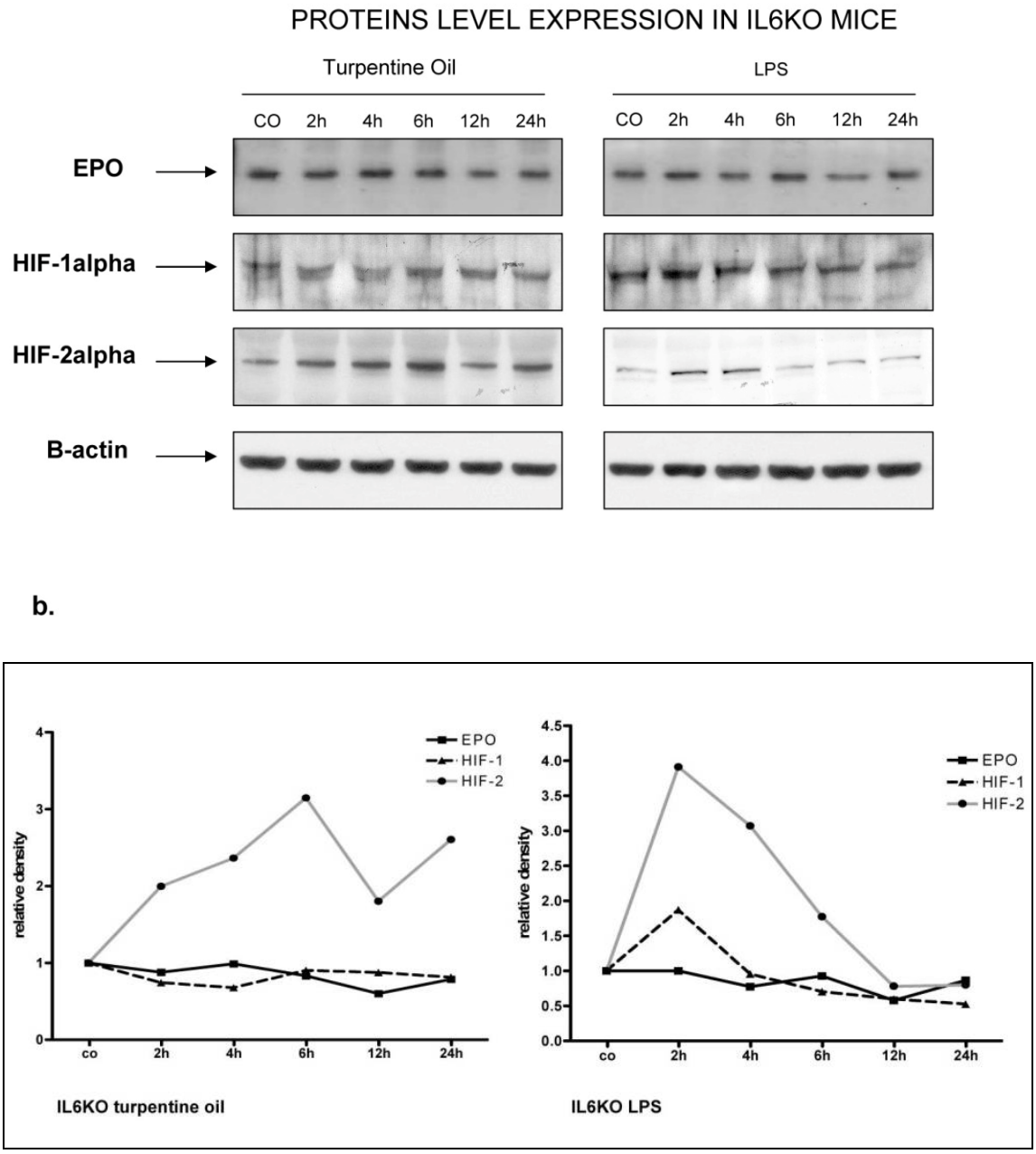


***Figure 16. Western analysis of EPO, HIF-1α and HIF-2α in liver of wild type (a) and IL6KO mice (b) during two different models of acute phase reaction.*** *In total cell lysates the liver content of erythropoietin, HIF-1α and HIF-2α was analyzed by western blot. EPO protein tissue levels results slightly increased in the liver between 4h and 12h. Although in mice the EPO tissue content resulted higher, a sudden release of the protein in the circulation and very low amount of stored protein in the cell could explain the low augment of signal detected by western blot. An earlier increase of the protein levels might be due to the infiltration of inflammatory cells from the circulation carrying pre-formed amounts of the protein. HIF-1α band resulted slightly increased at 6h after TO injection and strongly expressed between 2h and 6h after LPS treatment in wild type animals (**a**). IL-6KO mice submitted to TO treatment show no*

*evident increase of HIF-1α protein levels, whereas in LPS treated its expression resulted only reduced. The particular evident increase of HIF-2α in both models and for both strains indicates a protein regulation irrespective of IL-6 circulating levels (**b**). The relative density normalized with β-actin density showed under each western analysis better illustrates the changes of protein expression.*

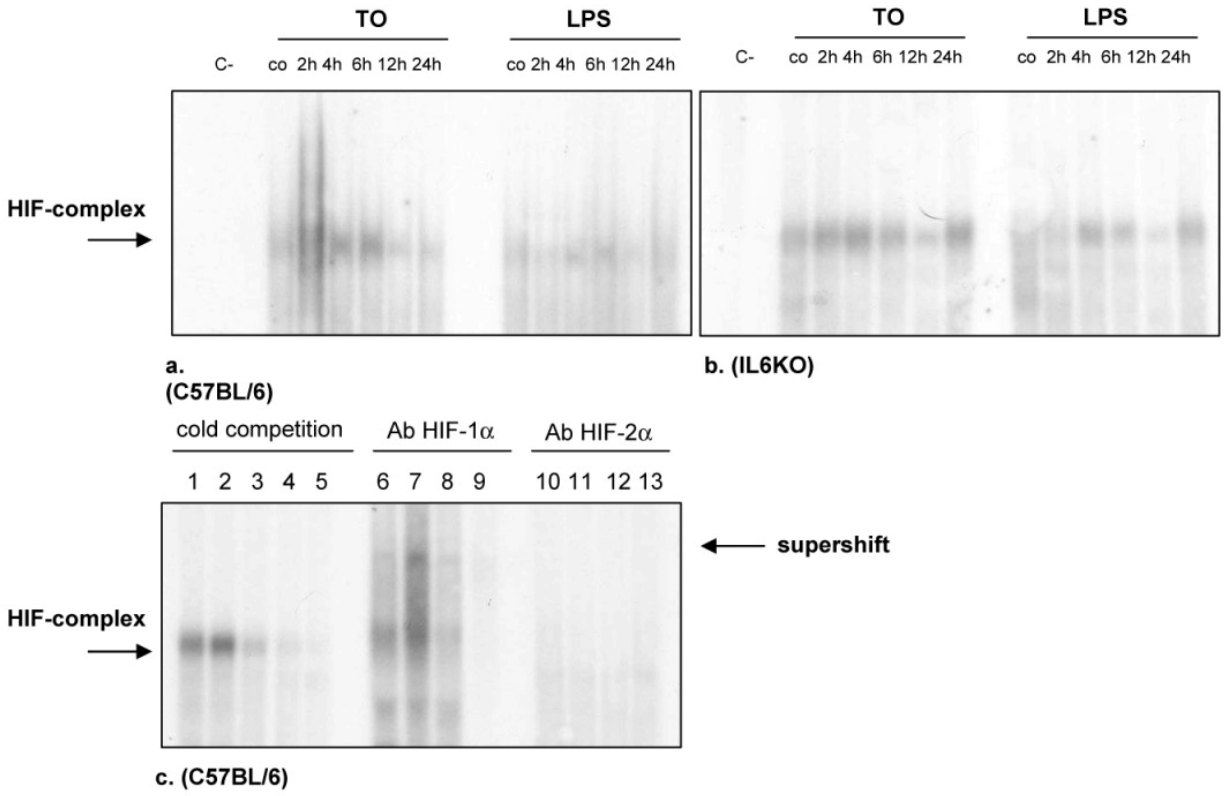


***Figure 17. EMSA analysis of HIF-DNA binding activity.*** *For the analysis of DNA binding activity an oligonucleotide containing the hypoxia responsive element sequence inserted in the erythropoietin enhancer was designed. The signal of binding results clearly increased between 4h and 6h in both acute phase models in the wild type strain, whereas a maximum of signal expression is recordable at 4h in the IL6KO strain (**a-b**). The supershift analysis confirms the involvement of both HIF members in the HRE binding region during acute phase reaction (**c**). (**C**: TO treated animals at 4h and 6h lanes, 1,2; competition at 6h with 100x, 200x and 400x cold probe, lanes 3-5; anti-HIF-1α 4h and 6h after TO, lanes 6-7, and LPS treatment, lanes 8-9; anti-HIF-2α 4h and 6h after TO, lanes 10-11, and LPS treatment, lanes 12-13). The specific binding of the probe to nuclear proteins was confirmed by competition with cold probe (**c**, lane 1-5).*

### *3.2.4 Stimulation with IL-6 induces an increase in EPO-gene expression and protein synthesis in hepatocytes, and up-regulates HIF-1α-gene and protein.*

From the *in vivo* studies a potential role for IL-6 in erythropoietin gene was confirmed. In order to support a regulatory role of this acute phase cytokine on EPO, HIF-1α and HIF-2α expression, a series of *in vitro* studies on isolated mouse hepatocytes was performed. The stimulation of mouse hepatocytes with increasing concentrations of IL-6 induced a significant up-regulation of EPO gene at a concentration between 10ng/ml and 100ng/ml of 2.5 and 1.5 fold respectively compared to the control group after 6h of treatment (fig.18a). Interestingly, we observed a dose-dependent induction of HIF-1α up to 2.3 fold increase but no effects were observed on HIF-2α gene (fig.18b-c). The bio-synthetic analysis in primary cultured mouse hepatocytes shows an increase in the protein synthesis after stimulation with IL-6 (single dose 100ng/ml culture medium) with an augmented signal in the supernatants too, to indicate an active release of erythropoietin in the culture medium (fig.18e). In parallel, a proportional increase of the HIF-1α protein levels was also observed in the hepatocytes after IL-6 stimulation, but no evident protein increase was reported for HIF-2α (fig.18d). Moreover, the immunostaining analysis also shows a nuclear accumulation of HIF-1α (fig. 21 upper panels) in the nuclei of hepatocytes treated with high concentrations of IL-6 (100ng/ml culture medium), but no evident effects on HIF-2α protein (fig.21, lower panels).

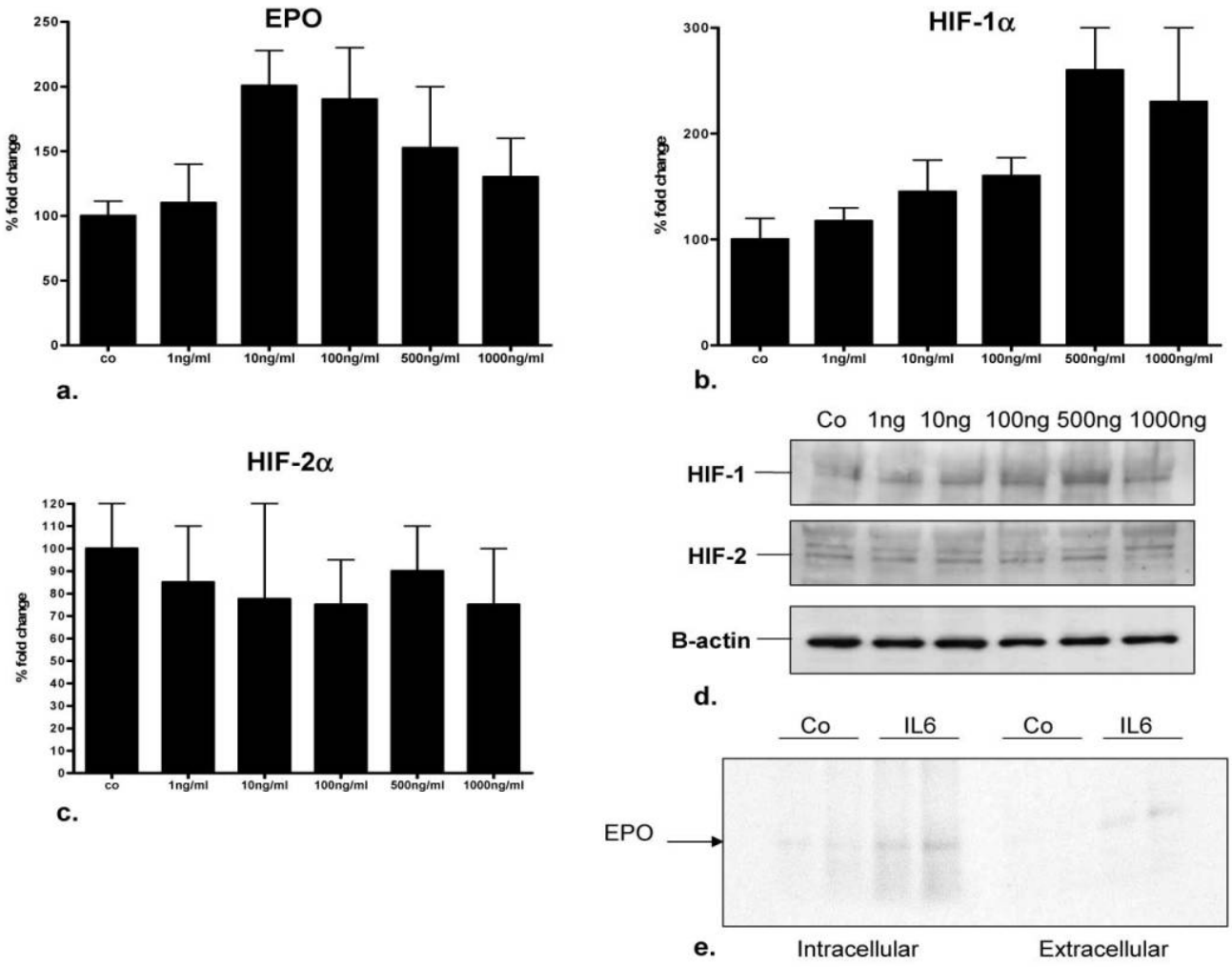


***Figure 18. Mouse hepatocytes stimulation with IL-6 in normoxic conditions.*** *Mouse hepatocytes isolated ex vivo from C57BL/6 mice were cultivated and growth for 24h before the treatments. Based on our previous observations on rat hepatocytes, IL-6 was used as main positive regulator of erythropoietin. The stimulation with different IL-6 concentrations at 6h induced a modest up-regulation of EPO gene at a concentration between 10 and 100 ng/ml and a dose dependent increase of HIF-1α gene expression (**a, b**), whereas no significant changes of HIF-2α gene expression were detected (**c**). The expression of the HIF-1increased particularly at concentrations between 10ng and 500ng/ml of IL-6 while HIF-2α protein levels remained almost unchanged in hepatocytes (**d**). Interestingly the immunoprecipitation following biosynthetic labeling reveals an increase of newly synthesized EPO in hepatocytes stimulated with 100ng/ml IL-6 for 6h, and an increase of the extracellular protein content indicating an active secretion from the hepatocytes (**e**).*

### *3.2.5 HIF-1α and HIF-2α tissue localization in murine liver during APR.*

From the data obtained by the western blot and by the bandshift assay a possible involvement of both HIF-1α and HIF-2α in the gene regulation during acute phase reaction turned out. Moreover, the experiments performed on isolated hepatocytes indicated a possible dominant cell specific expression of the HIFs members. In order to localize in the liver HIF-members protein expression an immunofluorescence staining was performed. The stain with anti-HIF-1α antibody resulted in a weak signal diffused to all the hepatic parenchyma, suggesting the presence of small amounts of the protein mainly in the cytosolic fraction of the hepatocytes in control animals. As reported in figure 19 after turpentine injection HIF-1α signal slightly increased and it is clearly localized in the nuclei of the hepatocyte although some extra-parenchymal cells (reasonably Kupffer cell) turned out to be positive too (fig.19b). On the other hand, the LPS model of acute phase reaction represents a phenomenon mainly driven by the macrophage compartment that directly interacts through the TLR4 receptor. In this case Kupffer cells evidently showed an increase of HIF-1α signal justifying the augment of the protein observed by western blot, although hepatocytes still expressed a stronger nuclear signal of fluorescence (fig.19c-d). The double immunofluorescence staining with the further addiction of a mouse macrophage specific antibody, F4/80, better localized HIF-1α expression in two different hepatic cell populations.

In the normal liver, the protein levels of HIF-2α were detectable mainly in the sinusoidal spaces (fig.20a), indicating an abundant expression mainly in endothelial cells and interstitial cell populations, like fibroblast and possibly Kupffer cells. Under normal physiologic condition hepatocytes revealed a weak presence of HIF-2α protein and even after TO injection and LPS treatment the clearest increase of signal was localized in endothelial cells and inflammatory cells (fig.20b-c). Interestingly, also neutrophils infiltrating the liver after LPS treatment and possibly activated Kupffer cells presented an evident positivity for HIF-2α (fig.20d).

A similar pattern of cellular expression was further confirmed by immuno-localization of HIF-1α and HIF-2α in different primary culture of liver cells population in vitro (fig.21-22). HIF-2α results in fact mainly localized in desmin-positive cells and isolated ED-2 positive cells, indicating a dominant localization overall in endothelial cells, fibroblasts and Kupffer cells (fig.22). Under normoxic conditions HIF-2α expression was weakly detectable in isolated hepatocytes (fig.21).

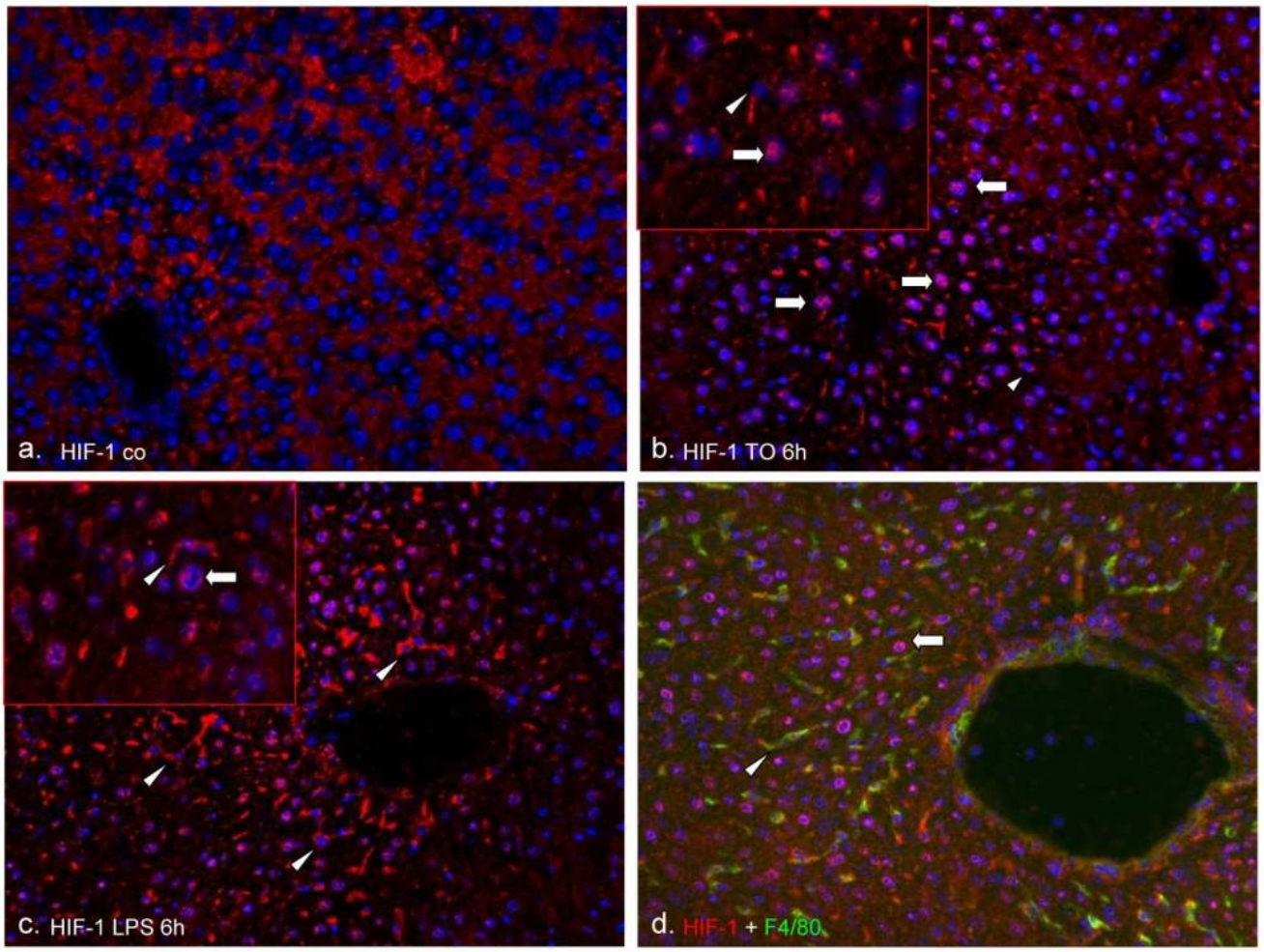


***Figure 19. Immuno-localization of HIF-1α in the liver of wild type mice during acute phase reaction.*** *Immunostaining analysis reveals a weak cytoplasmic positivity interesting all the hepatic parenchyma of control animals (a). In TO treated animals the positivity is particular evident in the nuclei of hepatocytes (b, arrows) with the presence of isolated extra-parenchymal positive cells (b,arrowheads). The LPS treatment (c) induces an increase of the*

*protein levels also in the non-parenchymal cell population (reasonably Kupffer cells from double staining, panel d in green, arrowheads) but the nuclei of hepatocytes predominantly express HIF-1α positivity. The double staining with HIF-1α and F4/80 identifies macrophages which also express the transcription factor (d, arrowheads).*

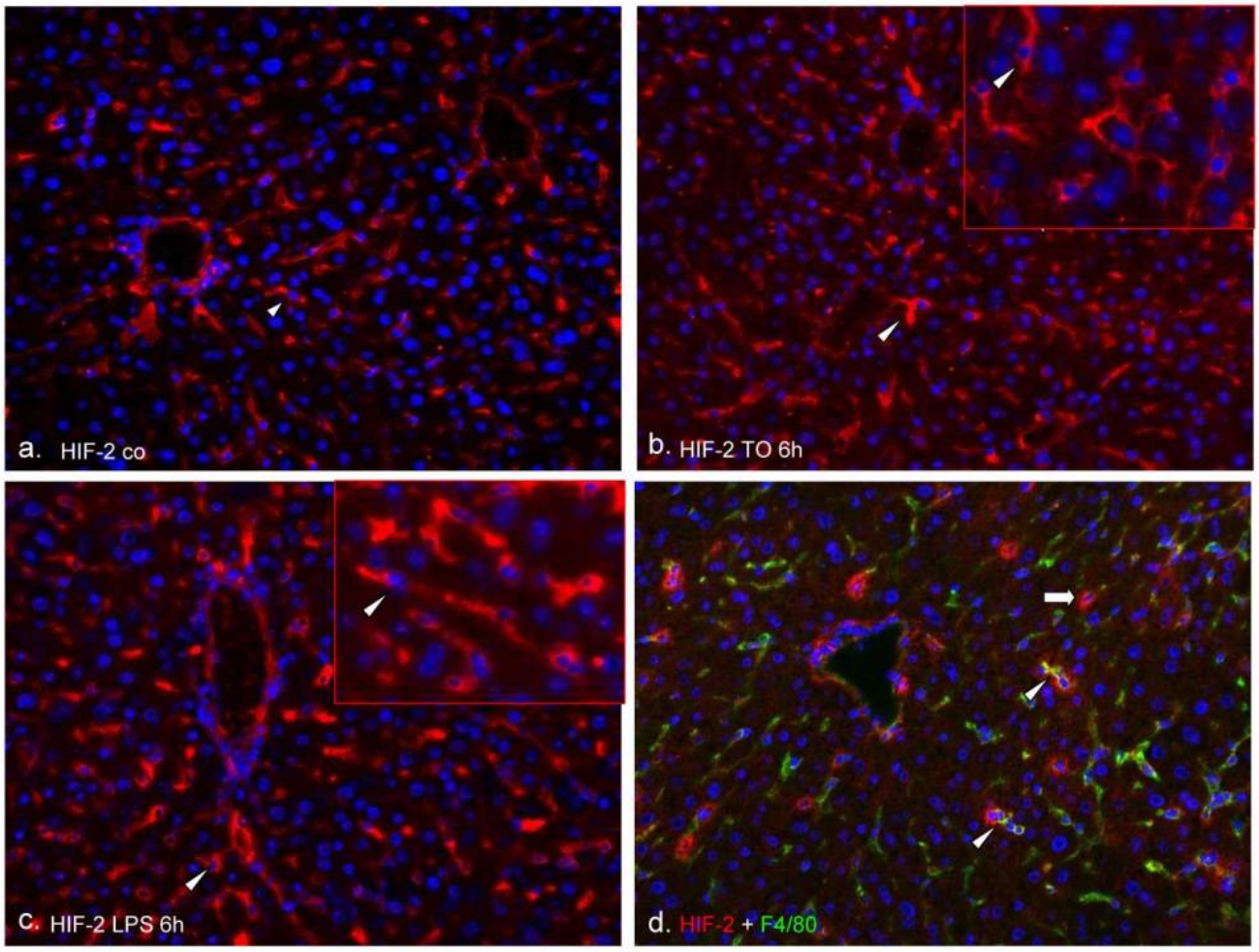


***Figure 20. Immuno-localization of HIF-2α in the liver of wild type mice during acute phase reaction.*** *HIF-2α immunostaining reveals the presence of the transcription factor mainly in the sinusoidal compartment, with a very weak positivity in the hepatocytes nuclei. The fluorescent signal increases after TO treatment in the extra-parenchymal district (sinusoidal areas) but also in some hepatocytes nucleus (b and d). Curiously, cells of the inflammatory infiltrate (granulocytes) particularly numerous after LPS treatment result positive for HIF-2α (arrows). The double stain (d)*

*reveals that macrophages also might express positivity for HIF-2α (red) in combination with F4/80 (green) (arrowheads).*

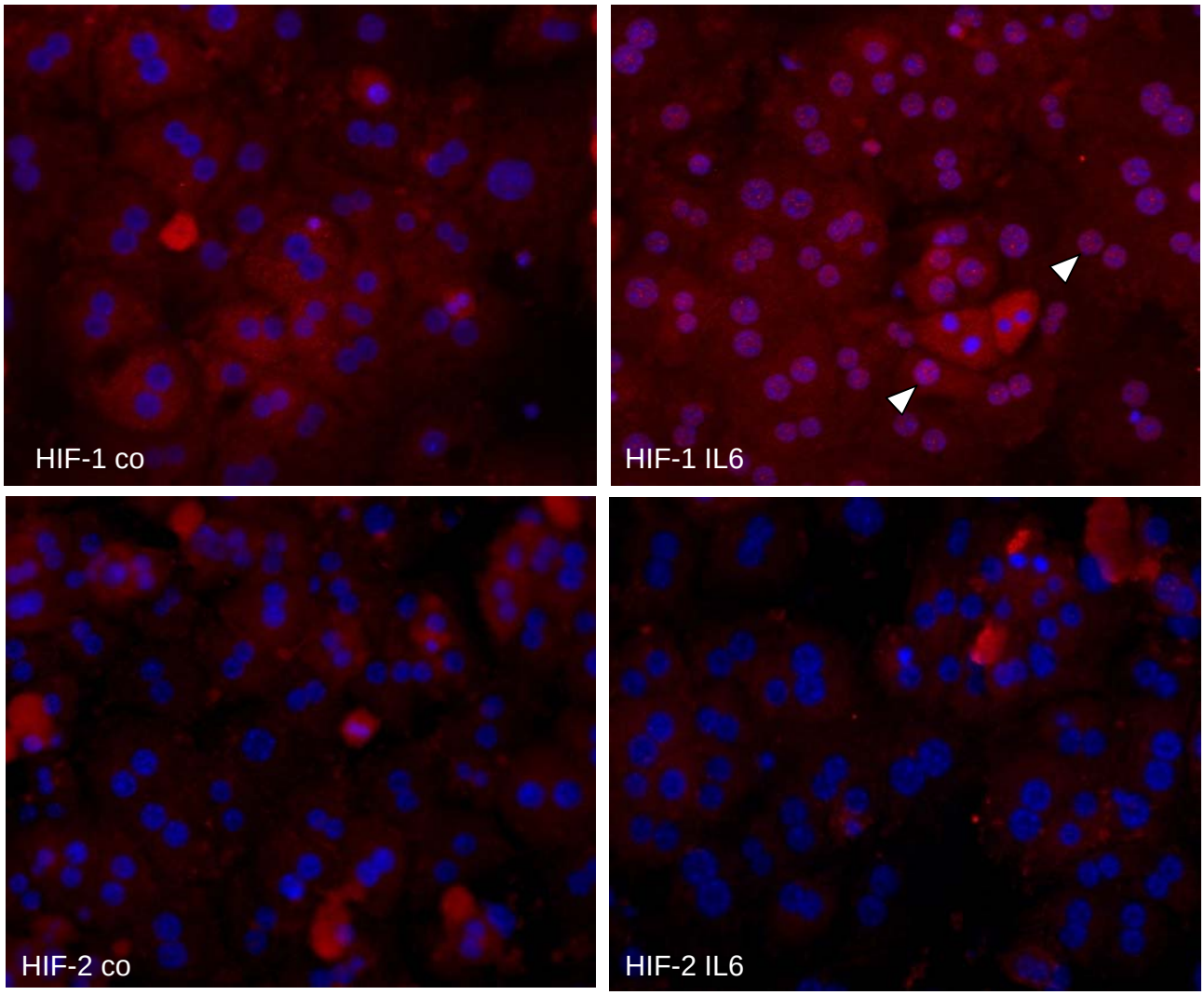


***Figure 21. Immuno-localization of HIF-1α and HIF-2α in mouse hepatocytes after IL-6 stimulation in normoxic conditions.*** *In normal hepatocytes very low levels of HIF-1α and HIF-2α protein are detectable. In order to investigate the role of IL-6 in the nuclear translocation of these two factors, mouse hepatocytes were challenged with a single high dose of IL-6. Although sporadic mouse hepatocytes present a weak positivity for HIF-1α, after the treatment with 100ng/ml of IL-6 a modest but clear signal in the hepatocyte nuclei was detectable (arrowheads). No changes or increase in HIF-2α positivity are detectable after IL-6 stimulation (magnification 200x).*

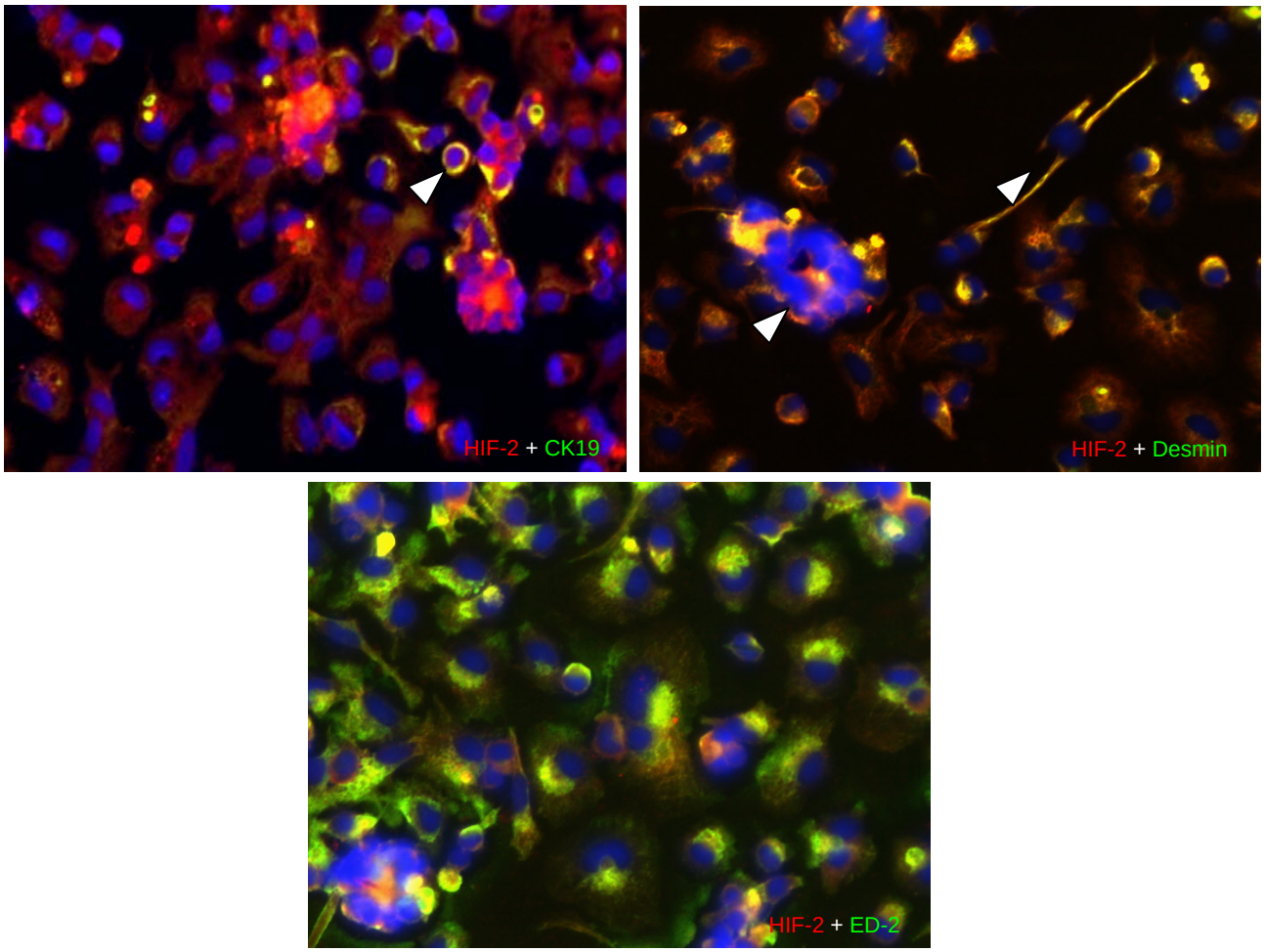


***Figure 22. Immuno-localization of HIF-2α in non-parenchymal liver cells in culture.***

*The previous immunocitological analyses on parenchymal cells indicated a preferential expression of HIF-1α in hepatocytes nuclei after IL-6 treatment. In order to localize HIF-2α expression in non-parenchymal cells, a double immunostaining was performed using HIF-2α antibody in combination with antibodies specific for other cell populations (anti-CK19 for cholangiocytes, anti- Desmin for fibroblasts and endothelial cells, and anti-ED2 for rat Kupffer cells). The isolation of a non-parenchymal cell fraction allowed the characterization of a macrophage-enriched cells population, with a residual contamination of less than 10% composed mainly by fibroblasts, and few endothelial cells and cholangiocytes. The stimulation with IL-6 (100ng/ml culture medium) seems not to influence significantly the levels of HIF-2α protein, expressed in modest cytosolic and nuclear amounts in control cells.*

*Regardless of the IL-6 treatment, it is possible to recognize small isolated round cells (arrowheads) or organized in clusters (arrows) that present a higher specific signal for HIF-2α (magnification 200x). The merge in the double-stain analysis revealed a main double-positivity for HIF-2α (red) and desmin (green) (arrowheads), indicating a probable identification of those cells as fibroblasts or endothelial cells, as also previously suggested, although CK-19 positive cells also express a marked positivity for HIF-2α.*

### *3.2.6 The expression of SOD-2, a gene known to be regulated by HIF-2α, increases during APR particularly after TO treatment and its increase resulted totally reversed in the IL-6KO strain.*

The mitochondrial SOD-2, together with EPO, has recently been identified to be one of HIF-2α target genes. We observed a significant up-regulation of SOD-2 gene with a peak of 3.4±0.5 fold increase at 12h after TO treatment in the wild type animals (fig.23a). No significant changes were observed in the IL6KO group. The LPS treated mice showed a lower augment in SOD-2 transcripts number and the peak of 2.5 fold increase registered in the wild type group at 4h preceded the one described by the IL6KO group at 6h without significant changes of expression (fig.23b).This data, together with the cell-specific localization, might support an involvement of HIF-2α as a redox sensitive transcription factor and its possible role in the control of the cell redox balance during acute phase reaction.

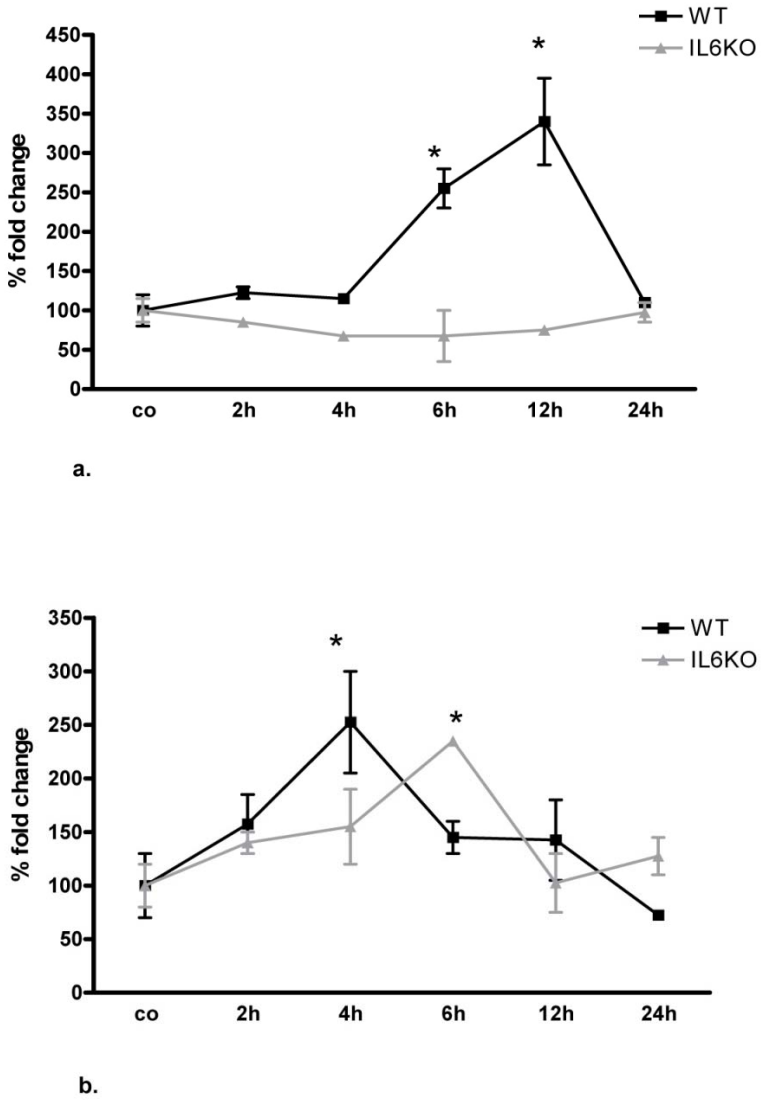


***Figure 23. SOD-2 mRNA hepatic expression in wild type and IL6KO mice after TO injection (a) and LPS treatment (b).*** *Superoxide dismuatase (SOD-2) is an antioxidant enzyme involved in the defense against free radical production. Its gene expression has been recently shown to be positively regulated by HIF-2α. In order to offer an example of a gene regulated in an erythropoietin-like manner we investigated its expression in the liver in the same conditions. The relative expression was normalized using β-actin as housekeeping gene. Similarly to the EPO gene kinetics, in the TO model, the expression of SOD-2 gene results significantly up-regulated at 6h after the injury to reach a peak of 3.4±0.5 fold increase in the wild type animals, whereas no significant changes are detectable (a). A different cellular and molecular pattern triggered by LPS administration reflects a different kinetic of expression with an increment of RNA transcripts up to 2.5±0.5-fold increase at 4h in the wild type group and up to 2.3±0.1-fold increase at 6h in the IL6KO group (b). Results are expressed as mean ± S.D. * Values significantly different compared with the control group (P<0.05, ** P<0.01 analyzed by Student's t-test, N=3).*

### *3.2.7 IL-6 in combination with IL-1β treatment induces opposite effect in EPO-gene expression in two different hepatoma cell lines, Hep3B and HepG2.*

The last analysis reported in figure 24 shows that the regulation of erythropoietin gene might be a complex cell-specific mechanism depending on different suppressors or activators. In spite of a similar induction of HIF-1α, the same cytokine or combination of cytokines might contribute to an increment of Epo-gene expression or to a decrement depending on the cell population analyzed (in this case Hep3B and HepG2 human hepatoma cell lines). This renders difficult to characterize a precise pathway controlling the Epo gene expression during normoxic conditions, although from this result its regulation appears not to be HIF-dependent. It is interesting to note that both hepatoma cell populations have been shown to be able to produce erythropoietin even during normoxic conditions (85). In particular, in steady state condition Hep3B cells, which in general share a more likely hepatocyte-like reaction to acute phase, express lower EPO mRNA levels compared to HepG2 but the gene expression resulted inducible following cytokines treatment.

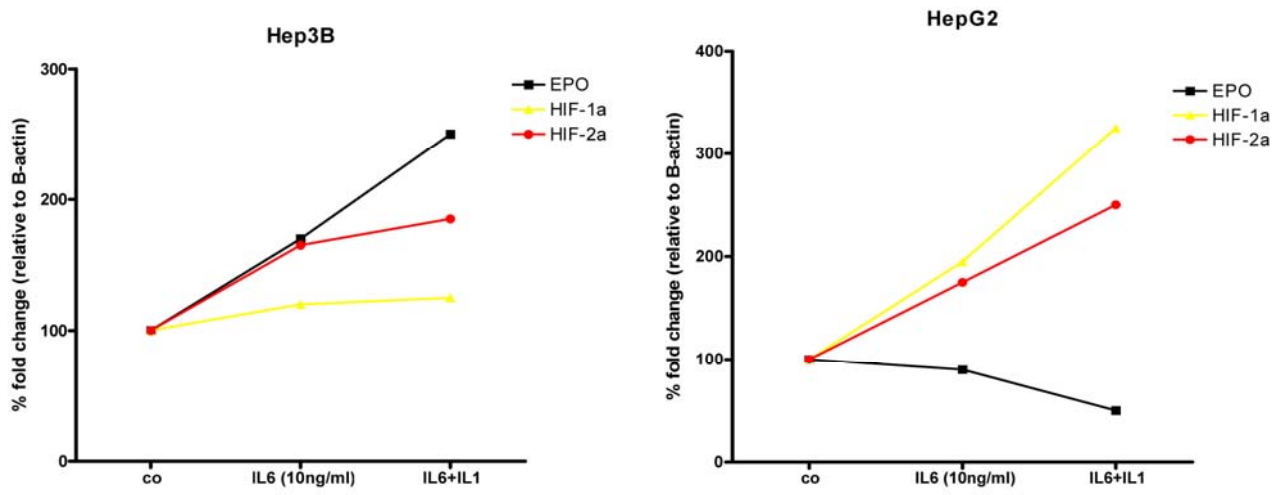


***Figure 24. EPO-gene expression in human hepatoma cell lines challenged with acute-phase cytokines.*** *On the base of previous analysis performed in hypoxic conditions on hepatoma cell lines suggesting a synergistic positive regulation of IL6 of erythropoietin gene, we analyzed the expression of EPO gene after stimulation with the same cytokine under*

*normoxic conditions. The relative expression was normalized using β-actin as housekeeping gene. In Hep3B (link) the stimulation with IL-6 and with an equal combination of IL-6 (10ng) + IL-1β (10ng) respectively induced a progressive up-regulation of the Epo-gene detectable after 6h. Similarly to the primary hepatocytes culture previously studied a light up-regulation of HIF-1α was recorded. Interestingly, in another human hepatoma cell line (right), expressing higher mRNA EPO levels in normal condition, the challenge with cytokines induced a down-regulation of Epo-gene particularly evident with a combination of IL-6+IL-1β, whereas HIF-1α and HIF-2α expression resulted progressively augmented. Furthermore the treatment with cytokines induced a stabilization of HIF-1α detectable by western analysis.*

# 4 DISCUSSION.

## 4.1 IDENTIFICATION OF ERYTHROPOIETIN AS AN ACUTE PHASE PROTEIN.

In a first part of the study, an increase of EPO levels was detected in the serum of rats treated with an intramuscular injection of turpentine oil, together with an increase of the mRNA and protein levels in the liver. The serum levels of EPO persisted significantly elevated up to 48 hours after the insult. Parallel to the increase of IL-6 serum levels, we observed a strong up-regulation of the EPO gene expression in the liver which progressively increased after the injection with a peak of expression between 4 and 12 hours. The treatment of isolated rat hepatocytes with IL-6, IL-1β and TNF-α offers a further confirmation that EPO might behave like a positively regulated acute phase protein. In fact, mainly IL-6 was capable to induce a transient and significant up-regulation of EPO gene expression.

Interestingly, a recent clinical study performed by Ferrucci et al. (86) revealed that patients with high values of circulating acute phase cytokines, e.g. IL-6, IL-1 beta, TNF-alpha, are more likely to develop a pre-anemic situation characterized by a dramatic increase of plasmatic erythropoietin. The authors explained this increase as a compensatory mechanism in response to changes in iron metabolism and a consequent reduction of hemoglobin synthesis as a consequence of inflammatory cytokine influence. Moreover, Tacke F et al. (87) reported an increase in plasma EPO concentration in patients developing chronic liver disease positively correlated to elevated plasmatic values of circulating IL-6. An analogue augment in serum EPO concentrations has also been reported in a murine model of subcutaneous inoculation of IL-6-producing cancer cells, but without effects on erythrocytes stimulation (88), as also observed in our model (data not shown). The authors attributed the lack of hemoglobin production and hematocrit induction to the insufficient levels of circulating iron and to disturbances of the iron metabolism, a typical situation also reported during

the acute phase response (89). An induction in normoxic conditions of hepatic erythropoietin in rats has also been described by Sohmiya and Kato after injection of growth hormone (GH) (90). Surprisingly, the authors observed an up-regulation of EPO gene and an increase of the protein content in the livers and plasma of the treated animals with a decrease of the renal production. A similar down-regulation of the EPO gene expression in the kidneys was also recorded in the experiments performed in the present study. However, previous analyses concerning cytokines effects on erythropoietin gene expression have been focus on kidney and hepatoma cell lines under hypoxic conditions.

Recent data published by Faquin and coworkers (91) about the effect of pro-inflammatory cytokines on hypoxia-induced erythropoietin production in a human hepatoma cell line Hep3B, showed that the pro-inflammatory cytokines IL-1alpha, IL-1 beta, TGF-beta and TNF-alpha were able to cause a dose-dependent inhibition of Epo, whereas IL-6 alone and a combination of IL-1β, TNF-alpha and IL-6 caused a dose-dependent induction. As suggested by the authors, the presence of a sequence highly similar to the consensus sequence for NF-IL6 in the 5' flanking region of the EPO gene could explain a prolonged induction of the gene in Hep3B cells observed 24 hours after stimulation with a combination of IL-6 and hypoxia. Similarly, the present study confirms the same results after treating the same hepatoma cell population with IL-6 and a cytokines combination, IL-6 with IL-1β, for a relatively shorter time of 6h under normoxic conditions. A slight up-regulation of the erythropoietin gene was observed in both conditions, but the expression resulted down-regulated after challenging with the same cytokines combination another hepatoma cell line, HepG2, also been shown to synthesize and release erythropoietin under normal conditions. This observation complements with a previous study on HepG2 cells performed by Hellwig-Buergel and colleagues (92), who showed that IL-1 beta and TNF-alpha stimulation induces an increase of HIF-1α protein expression and of its binding activity even in normoxic conditions, but without up-regulating Epo-gene expression. For the first time, an acute and transient induction of EPO up to 12 hours was described in this study in isolated rat hepatocytes exposed to IL-6 treatment under normoxic

conditions, but a dose-dependent inhibition after TNF-alpha treatment. By the *in vitro* analysis performed in the present and in previous works emerges that erythropoietin gene expression results differently regulated in different cell-types indicating alternative mechanisms of gene control cell-specific parallel to the oxygen-dependent pathway.

Because of the complexity of the system and because of the multitude of factors involved, the *in vivo* regulation of the erythropoietin gene is still partially unknown. Most of the studies concerning transcriptional regulation of the HIF family through conditional inactivation of the single members indicates HIF-1α as a major stimulator of the expression of glycolytic enzymes, e.g. carbonic anidrase-IX, PGEK, LDH-A (93), while HIF-2α has been shown to positively regulate the EPO gene during hypoxic and physiologic conditions in the liver (94). Furthermore, HIF-2alpha has recently been attributed a relevant role in the control of intestinal iron adsorption and distribution, with effects on the modulation of several hepatic genes (95). A growing body of evidence (96, 97) indicates a binding sequence for STAT-3 in HIF-1α gene promoter, but HIFs activity seems to depend mainly on protein stabilization and post-translational modifications without direct correlations with the gene expression (98). In fact, on the one hand an increase of HIF-1α mRNA expression was detected between 2 and 6 hours contributing to a progressive accumulation of the protein up to 24 hours after the inflammatory insult, but on the other hand HIF-2α RNA expression did not result to be related to the protein increase, especially in the rat model. Although the RT-PCR analysis supports a possible role of STAT-3 pathway in the HIF-1α gene up-regulation, the protein stabilization for both transcription factors could depend on other pathways triggered by different cytokines or mediators involved in the phenomenon.

A cooperative association between the pro-inflammatory transcription complexes NF-kB and HIF-1α has been suggested as a common point between two inseparable processes reciprocally bound to each other, like inflammation and hypoxia (99, 100). In particular, cooperation between the NF-kB essential modulator (NEMO) and HIF-2alpha is thought to specifically increase the activity of this

transcription factor even under normoxic conditions (101) and could be responsible for the increase in protein stabilization observed in the models presented in this study.

This first part of the work suggests that during acute phase response erythropoietin might behave like a positive acute-phase protein and the liver represents the major source of this hormone in this specific physio-pathological condition. Moreover, its raise parallels the induction of HIF-1α and HIF-2α, its main transcriptional regulators. A detailed analysis of EPO-gene expression in experimental models of acute and chronic liver injury (CCl4 treatment, partial hepatectomy, bile duct ligation-induced cholestasis, thioacetamide intoxication) and in experimental hepatic cancer models analyzed during the development of the present work revealed an up-regulation concomitant with the increase of IL-6 circulating levels and with a recovery from tissue damage. As EPO has been shown to exert an antioxidant and proliferative effects in different tissues, such as in the brain and in several models of tissue injury (102), its hepatic up-regulation during acute phase response could be independent of its hematopoietic functions.

## 4.2 IL-6 PLAYS A KEY ROLE IN THE HEPATIC ERYTHROPOIETIN EXPRESSION DURING ACUTE-PHASE REACTION INDEPENDENTLY OF HIF-MEMBERS ACTIVATION.

On the basis of the previous observations (103), a successive step aimed at the investigation of the mechanisms regulating EPO-gene expression in the same normoxic conditions in absence of IL-6, a fundamental cytokine involved not only in the early phases of the acute-phase response but also in the resolution of the inflammatory process. In addition, the same analysis was performed in terms of a comparison of two different murine models of acute phase reaction in order to studies the combination of different acute phase mediators on the hepatic gene-expression *in vivo*.

A first consideration emerging from this second part of the study can be deduced by the observation that the hepatic contribution up to a 40% of the total body EPO production has been clearly

illustrated in rats whereas in mice and sheep the hepatic contribution of the total body EPO production has been shown to reach only a 15-20 % in hypoxic conditions (104). This specie-specific difference could explain a delay in the hepatic EPO mRNA increase observed between rats and mice after an acute-phase stimulus. Differences in the pattern of erythropoietin induction were also previously reported in terms of tissue specificity (kidney-liver) and cell specificity (hepatocytes-Kupffer cells, or Hep3B-HepG2). Epo mRNA expression increase in the murine liver during APR results detectable between 6h and 12h after the injections in parallel to an augmented activity of the two main transcription factors involved in EPO-gene regulation, HIF-1α and HIF-2α. The lack of IL-6 did not influence HIF-2α protein expression, thus HIF-1α mRNA expression resulted strongly reduced and the hepatic gene expression and serum concentration of erythropoietin dramatically declined in the IL6KO mice after the insults. Considering two different experimental conditions, this result seems to be in contrast with a recent publication by Rankin and co-workers (94) demonstrating with the use of conditional gene inactivation and von Hippel-Lindau tumour suppressor deficient mice a crucial role of HIF-2α in the positive regulation of the hepatic erythropoietin in mice during hypoxia. In support of our in vivo results, the stimulation of isolated mouse hepatocytes with several concentrations of IL-6 once more confirmed the EPO-gene up-regulation but, importantly, a *de novo* synthesis and release of the protein was detected through biosynthetic labelling, demonstrating a direct regulative function of this important acute phase cytokine on hepatocytes cultured in normoxic conditions.

The regulation of erythropoietin-gene expression during hypoxia has been widely explained (105), but from Rankin's work emerged that HIF-1 and HIF-2 can activate simultaneously distinct target genes implicated in the cell adaptation to hypoxia. The HIF transcription factors family has been initially identified as the most important regulator of EPO-gene under hypoxia and in the adult kidney (106), and the specific involvement of the single members of this transcription family in the direct gene activation is still an opened question (107). In these two models of acute phase reaction, a hepatic increase in the protein levels of both members came to surface and the super-shift analysis

performed in this work revealed a possible involvement of both members of transcription factors suggesting a contemporary activation in the same pathologic conditions. The identification with immunofluorescence revealed a dominant positivity for HIF-1 in hepatocytes and isolated clusters of Kupffer cells, whereas HIF-2α resulted dominantly expressed in endothelial cells and other non-parenchymal cells, possibly fibroblast-like cells, with a weaker nuclear induction of protein levels in hepatocytes during inflammation. It could be interesting to mention that Rankin et al performed their study in PEPCK-Cre transgenic mice, gene that is also well expressed in non-parenchymal cell populations such as Kupffer cells (data not reported in this work). These observations support two concepts that require future investigations: a possible contribution of the non-parenchymal compartment in the EPO production and a cell-specific balance between transcription factors that according to the cellular microenvironment might alternatively influence the regulation of the same gene.

On the basis of Rius et al (108) and Bracken CP et al (109)'s work introducing an essential association between HIF-1α and HIF-2α respectively with the transcription factors family NF-kB, Taylor elegantly reviewed an interconnection between these transcription factors in a multi-factorial complex machinery (110). Beyond NF-kB, it has been proposed in past studies that STAT-3 may be able to stabilize HIF-1α in an oxygen-independent manner, competing with pVHL for the binding to the transcription factor decreasing in this way its ubiquitinilation and successively its degradation (111). The result of this activation seems to trigger different pathways depending on the member associated to HIF: in fact whereas IL-6 appears to induce Epo gene up-regulation as illustrated in the present work, on the other hand NF-kB resulted in an inhibition of Epo transcription (112). Whereas the prevalence of a STAT3-HIF-1α complex could elicit Epo gene induction, the dominance of NF-kB in association with HIF-1α might result in an inhibition of Epo gene expression due to allosteric competition as previously shown. Comparing the graphs in figure 1 and figure 10, this hypothesis could reasonably explain the temporal difference of Epo induction observed between rats and mice. In fact, in rats the values of IL-6 are drastically higher compared to

IL-1β already in earlier time points and the values of Epo gene in the liver increment from 2h after injury. In the mice the difference between IL-6 and IL-1β expression is relevant only at 6h, time point in which the Epo gene expression start to increase.

A crucial role for IL-6 in the erythropoietin production in hepatocytes during acute phase response and in the hepatic HIF-1α mRNA up-regulation clearly emerged. As recently reviewed by Semenza (113), HIF-1α controls the absorption and delivery of iron to the bone marrow, process mediated through the regulation of at least 5 different gene products (EPO, EPOR, hepcidin, transferrin, and transferrin receptor) involving 5 different organs (kidney, liver, intestine, blood and bone marrow). The importance of HIF-1α is not only limited to the macrophage compartment in the regulation of the innate response, but its dominant expression in hepatocytes during acute-phase might delineate a novel function of HIF-1 in the control of acute-phase mediators. Although HIF-2α resulted increased in both the model, indicating a participation of this transcription factor in the inflammatory process, no evident correlation with IL-6 was observed. The transcription factor NF-kappaB has been presented as a positive regulator of HIF-2α and as a redox-sensible transcription factor. A potential role of HIF-2α in the reactive oxygen species-mediated signalling has been recently proposed (114) and it is interesting to note that in cells particularly exposed to redox alterations, like granulocytes and activated endothelial cells, HIF-2α expression resulted particularly relevant. Under hypoxic conditions, characterized by dramatic alterations of the mitochondrial respiration and a massive release of oxygen reactive species, the transcription factors balance might decline in favour of HIF-2α-dependent gene regulation even in hepatocytes. Curiously, SOD-2 (manganese superoxide dismutase), another gene supposed to be regulated also through HIF-2α, that was up-regulated in parallel to EPO in the wild type groups, showed no induction in the IL6KO mice after TO-injection, but the LPS treatment showed a SOD-2 gene induction also in absence of IL-6.

IL6 knockout mice submitted to two different experimental models of acute-phase reaction showed a dramatically reduced gene expression and serum concentration of EPO compared to the wild type groups, independently on the activation of the HIF transcription factor members. The comparison between two different models of acute response triggering different pathways of signalling brought to surface also a possible cell-specific EPO-gene regulation in distinct physio-pathological settings. Hepatocytes, capable to newly synthesize and release erythropoietin following IL-6 stimulation, may represent the major producers of Epo in the liver through a possible direct regulation of this cytokine. As depicted in figure 25, a further contribution from other cell populations might come following a disruption of the cellular redox balance that characterizes the inflammatory processes possibly influencing HIF-2α activity. The identification of a key role for IL-6 in the regulation of the EPO gene expression during acute phase reaction might give a further contribution to the understanding of the hypoxia-related immune response.

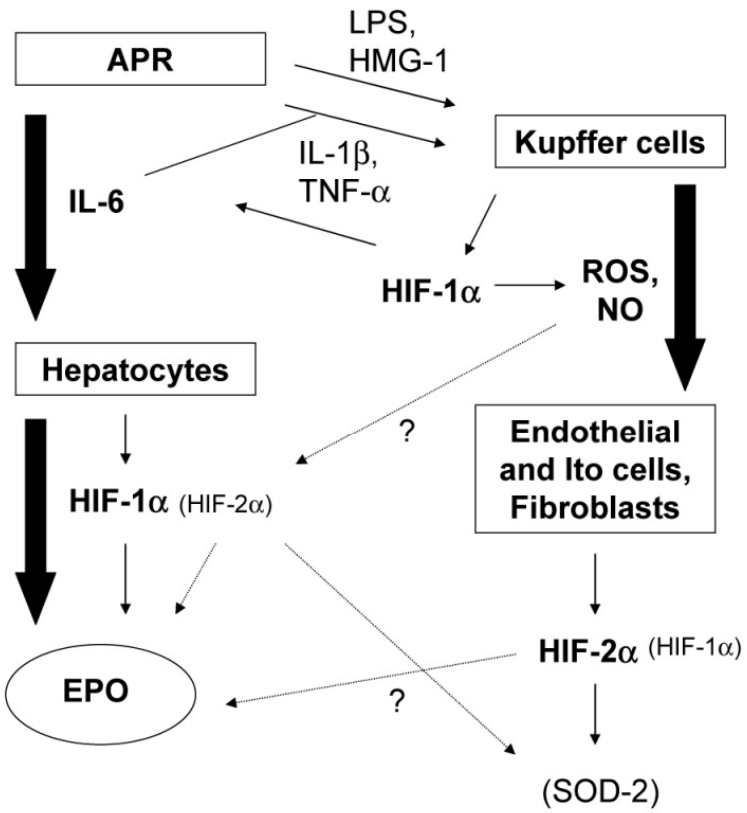


***Fig. 25. Possible mechanisms of EPO, HIF-1α and HIF-2α circuitation in the liver during acute phase response.*** *The acute-phase response act on the hepatocytes mainly through the mediator IL-6, in which it triggers to the release of acute phase proteins and EPO (big black arrows). On the hepatocytes IL-6 has also been shown to induce HIF-1α expression and its nuclear translocation, possibly related to EPO production (arrows). IL-1β, TNF-α, LPS and the homeo-box (HMG-1), are all acute phase mediators that predominantly act on Kupffer cells triggering to the activation of HIF-1α which regulates in return the expression of the same cytokine and the phagocytic activity of those resident macrophages inducing the production of reactive oxygen species and nitric oxide (ROS and NO, arrows). The ROS and the cytokines produced by Kupffer cells could participate to the activation of endothelial cells and fibroblast-like cells inducing the expression of HIF-2α through the redox sensible transcription factor, NF-kappaB (big black arrows). The alteration of the cellular redox environment might offer a further contribute to the EPO production via HIF-2α activation in fibroblast-like cells and hepatocytes.*

## 4.3 CONCLUSIONS AND FUTURE PERSPECTIVES.

The results obtained in this work mainly suggest two new concepts concerning the expression and the regulation of Epo gene. First, erythropoietin behaves as an acute phase protein and its induction and release is described in this work in two different experimental models, septic and aseptic acute reaction, under normoxic conditions. As the liver represents one of the main target organs of acute phase cytokines and the major source of acute phase proteins regulating the entire physio-pathological process, the hepatic parenchyma represents the major source of erythropoietin in these specific conditions. Although individual and specie-specific differences depict different patterns of gene expression, the renal source responsible for Epo production during physiologic and hypoxic conditions appeared to be inhibited during acute phase response in favor of the hepatic source. The meaning of Epo release during acute phase reaction might reasonably be linked not only to compensative mechanisms counteracting the metabolic changes interesting the acute-phase but also attributed to anti-apoptotic and anti-inflammatory functions involved in the resolution of the inflammatory process. Future studies might aim at the identification and functions of the Epo-EpoRec pathway during acute liver tissue injury and in the survival of different models of hepatic carcinoma as preliminary data already indicate.

The second important information emerging from this work concerns the regulation of Epo gene expression during acute phase response that appears to depend mainly by IL-6. Indeed, the lack of this cytokine resulted in a progressive loss of circulating Epo under these conditions and in a dramatic reduction of the hepatic gene expression. The identification of an important role for IL-6 in erythropoietin gene positive regulation suggests alternative pathways that might influence the gene transcription under normoxic as well as under hypoxic conditions. This could represent a scientific basement for further investigations aiming at the identification of IL-6 responsive elements on erythropoietin gene enhancer or promoter. Although HIF-1$\alpha$ protein has been shown to be stabilized and activated also by other cytokines such as TNF-$\alpha$ but without altering erythropoietin gene expression, a luciferase assay might be useful to clarify a direct activation of

HIF-1α transcription factor through IL-6 dependent pathways, as previously hypothesized in various cancer cell lines. The results obtained in these acute conditions might explain the crucial role played by the axis IL-6-STAT3-HIF-1α shown to be critical in several cancer cells survival and proliferation and characterizing the tumor-related immune microenvironment. The response of erythropoietin gene expression to hypoxia in IL-6KO mice could be a successive study in order to clarify the role of this cytokine in the hypoxia-related immune microenvironment.

Interestingly, HIF-2α has been recently suggested as the main positive regulator of Epo gene expression in the liver during hypoxia, but the loss of IL-6 did not affect the increase of expression of this transcription factor observed during acute phase reaction. A further message emerging from this study is that HIF-1α and HIF-2α resulted both augmented after acute phase, but with different cellular localization. The heterogeneity of the hepatic cell population rendered quite difficult the identification of cell-specific Epo producers even during hypoxic conditions, and this point still results under analysis. A cell-specific analysis of transcriptional regulation in the different cell population could bring to the identification of erythropoietin production in different organs, under different stressful conditions and with different functions.

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