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Malaria-Induced Apoptosis in the Cells of Interlukine-6Rα knockout Mice

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Abstract: The purpose of this study was to illustrate the function of IL-6Rα during malaria infection by investigating the responses of *Plasmodium chabaudi* malaria in hepatocytes and spleenocytes of IL-6R$\alpha^{+/+}$ and IL-6R$\alpha^{-/-}$ female C57BL/6 mice via evaluation of mRNA expression of apoptotic genes (Bax, Bcl-2 and Caspase-3). Mice were injected intraperitoneally (ip) with 10^6 *P. chabaudi*-infected erythrocytes and then scarified at days (0, 1, 4 and 8, respectively). RT-PCR was used to quantify apoptotic genes level. In the IL-6R$\alpha^{+/+}$ the levels of liver Bax were significantly increased at days 1 and 8 while they were significantly decreased only at day 1 in the IL-6R$\alpha^{-/-}$ compared with day 0. In contrast, the IL-6R$\alpha^{+/+}$ levels of spleen Bax were significantly increased only at day 8. In the IL-6R$\alpha^{+/+}$ the levels of liver Bcl-2 were significantly increased at all days after infection while significantly decreased only at day 1 in the spleen IL-6R$\alpha^{-/-}$ compared with day 0. In the IL-6R$\alpha^{+/+}$ the levels of liver caspase-3 were significantly increased at days 1 and 8 while significantly decreased in the IL-6R$\alpha^{-/-}$at day 8 compared with day 0. However, in the IL-6R$\alpha^{+/+}$ the levels of spleen caspase-3 were significantly increased at day 8 only while significantly decreased at day 8 in the IL-6R$\alpha^{-/-}$compared with day 0. In conclusion, this data has shown that the absence of IL-6Rα plays an important role in decreasing the severity of apoptosis during malaria disease. Thus, this study suggests that after further investigation the role of IL-6Rα could be the therapeutic key for determining the outcome of malaria in liver cells.

Keywords: Apoptosis, IL-Rα and spleen, liver, malaria, mice

INTRODUCTION

Malaria is one of the most dangerous infectious diseases and is considered endemic in many tropical countries. About 120 million people each year suffer symptoms of this disease (Alkahtani *et al*., 2011). According to the World Health Organisation (WHO, 2005), malaria is one of the world's fourth leading causes of death in children under five and about 400 million people suffer from malaria worldwide with about 1.5 million deaths per year (Greenwood *et al*., 2005; Roberts *et al*., 2001). The available anti-malaria drugs are ineffective because of the resistance of malaria (Carlton *et al*., 2001). More investigations are required to understand the protective mechanisms of the host and pathological complications induced by parasite (Callaway, 2007). Mice are either resistant or susceptible to blood stages with *P. chabaudi* malaria, which is controlled by both genes of the mouse MHC, the *H2*-complex and genes of the non-*H2* background as well as diverse endogenous soluble factors (Roberts *et al*., 2001; Wunderlich *et al*., 1988).

Liver has a potential role in malaria infection. For example It is the site of preerythrocytic development of *Plasmodium* parasites and also acts as an effector against malarial blood stages (Krücken *et al*., 2005b; Wunderlich *et al*., 2005). About 90% of Kupffer cells are able to eliminate Plasmodium-derived hemozoin. Later on, Kupffer cells activated produce the NO (nitrogen oxide), diverse ROS (radical oxygen species) and the cytokines IL-1β and TNFα. Both, NO, ROS and the cytokines result in enormous pathological complications in the liver (Alkahtani *et al*., 2011). Indeed, IL-1β and TNFα are thought to induce the acute phase response and a dramatic decrease in liver metabolism, both primarily based on liver hepatocytes. The NO and ROS in turn induce an oxidative stress which results in an increase in apoptosis of hepatocytes (Akanmori *et al*., 1996; Krücken *et al*., 2005a) On the other hand, apoptosis plays an important role in liver pathology such as in the dysregulation of the infected liver. This is emphasized by the fact that malarial blood stages also induce the production of IL-6 (Akanmori *et al*., 1996; Gourley *et al*., 2002). This pleiotrope cytokine is generally regarded as protective in liver injury (Barton, 1996) and IL-6 is widely known for its inhibitory action in apoptosis, i.e., IL-6 appears to be beneficial for malaria, since it counterregulates the malaria-induced apoptosis in liver hepatocytes.

The effect of IL-6 on liver cells comes through the IL-6R (Barton, 1996). The IL-6R complex consists of the ligand-binding subunit glycoprotein (gp80 = the α-subunit) and the signal-transducing subunit gp130. Binding of IL-6 to membrane-bound gp80 leads to recruitment of the signal transducing subunit gp130, which then activates JAK and finally STAT factors, which in turn are involved in the control of apoptosis as well as other cellular processes (Barton, 1996). On the other hand, the spleen is believed to participate in cleaning parasites from the circulation and providing a strong hematopoietic response during acute infections (Dkhil, 2009).

Cytokines are inflammation mediators of the immune system and belong to a group of short-lived polypeptides (20 kDa) which exert a modulating effect on cells of the innate and adaptive immune system. So cytokines control and coordinate the defence against pathogens. In the context of an inflammatory response to infection, an interleukin-6 release leads to the activation and differentiation of Immune cells. Here, IL-6 acts as a Hepatocyte Stimulating Factor (HSF) which is active across a wide spectrum, such as in the regulation of hematopoiesis (Alkahtani *et al*., 2011).

As is well-known, apoptosis is an active form of cell death closely bound up both pathologically and physiologically with a variety of cellular systems. Apoptosis may occur via a death receptor-dependent (extrinsic) or independent (intrinsic or mitochondrial) pathway. The death receptor pathway comprises Fas (CD95/Apo-1) and TRAIL (Apo-2) and this pathway is activated when ligands specific for either Fas or TRAIL bind to their respective receptors, resulting in activation of caspase-8, which finally activates caspace-3 (Alkahtani, 2011). In addition, mitochondrial pathway of apoptosis is initiated by the down-regulation of anti-apoptotic proteins (such as, Bcl-2, Bcl-xl) and/or up-regulation of pro-apoptotic proteins (such as, Bax, Bad, Bid), causing opening of mitochondrial permeability transition pores and release of apoptosis inducing proteins (cytochrome c, apoptosis inducing factor etc.) from mitochondria (Alkahtani, 2011).

Until now, the role of IL-6 in malaria disease has not been understood. So this study was undertaken to investigate whether IL-6 has any effects on the liver and spleen mice cells during malaria infection and-if so-as to whether IL-6 interferes with the extrinsic and/or intrinsic pathway of apoptosis.

MATERIALS AND METHODS

Animals and infection: Mice with disrupted (IL-6$\alpha^{-/-}$) have been previously generated by Professor Thomas Wunderlich, University of Cologne, Germany. The same mixed genetic background is present in the *w.t*$^{+/+}$mice. Normal C57BL/6 laboratory female mice 10-12 weeks old and weighing 23-36 g were used throughout the study and housed in plastic cages. Animals maintained under the usual standard laboratory conditions at a temperature of 22±1ºC, a relative humidity of 45±5% and photoperiod cycle of 10/14 h. Mouse food (commercially available) and water were offered *ad libitum*.

Blood stage malaria: Erythrocytic stages of *P. chabaudi* were injected once a week in both wild type and IL-6α knockout mice. From infected mice, blood was taken from the tip of the mouse tail and 10^6 *P. chabaudi*-infected erythrocytes were injected i.p. in the mice. Parasitemia was evaluated by using Giemsa-stained blood smears. The number of erythrocytes was determined by light microscope. Five mice of each group were killed at each day (0, 1, 4 and 8, respectively).

RNA-isolation: Two hundred and fifty mg of frozen liver and spleen were homogenized for 1 min then mixed with 1 mL chloroform for 15 sec. The suspension was incubated for 15 min at room temperature and centrifugated at 3.000 x g for 45 min. Isopropanol was used for precipitation of the supernatant; the pellet was washed twice with 80% ethanol, dried and then dissolved in 200 μL RNase-free water. RNA concentrations were determined at 260 nm and the purity of RNA was checked in 1% agarose gel.

Quantitative real-time PCR: All RNA samples were treated with DNase for 1 h and then converted into cDNA. Quantitative RT-PCR was conducted by using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Mastermix from Qiagen (Hilden, Germany). The Gene expression were investigated via mRNA for following proteins: 18S, Bax, Bcl-2, Caspase-3. The primers used for qRT-PCR were obtained from Qiagen. PCR reactions were conducted as follows: 2 min at 50 and 95ºC for 10 min, 40 cycles at 94ºC for 15 sec, at 60ºC for 35 sec and at 72ºC for 30 sec, respectively. Gene 18S rRNA was used as control. The induction of mRNA expression on days (0, 1, 4 and 8 *p.i.* was determined by using the $2^{-\Delta\Delta ct}$-method (-ΔΔct = Δct day 0 *p.i.*-Δct day 8 *p.i.*).

Statistical analysis: The two-tailed Student's t-test and Fisher's exact test were used for statistical analysis.

RESULTS

Characteristic of *P. chabaudi* infection: Infected mice with 10^6 *P. chabaudi*-infected erythrocytes became evident on day 4 reaching a peak of 48% on day 8 then falling rapidly to about 0.4% on day 12 (Fig. 1).

Apoptotic genes expression: Quantitative real-time PCR was used to detect changes in mRNA levels of different apoptotic genes in the liver and spleen of both wild type

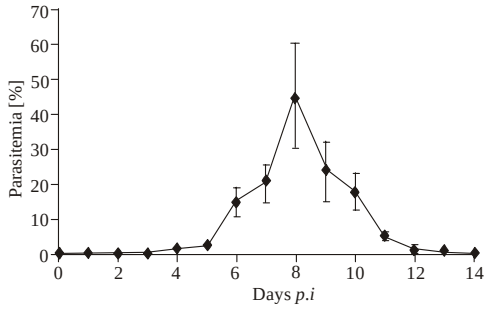

Fig. 1: Parasitemia (48%) of female C57BL/6 mice (n = 20) infected with 10^6 *P. chabaudi*-infected erythrocytes

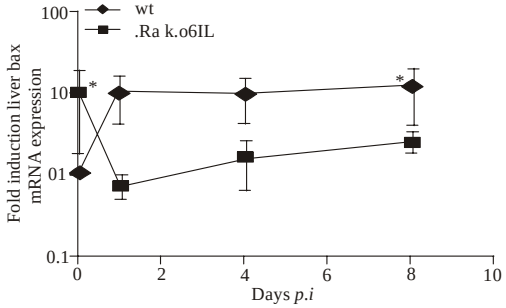

Fig. 2: RT-PCR of Bax gene expression in the liver of *Plasmodium chabaudi* infected IL-6Rα$^{+/+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5), *: significant value at (p<0.05)

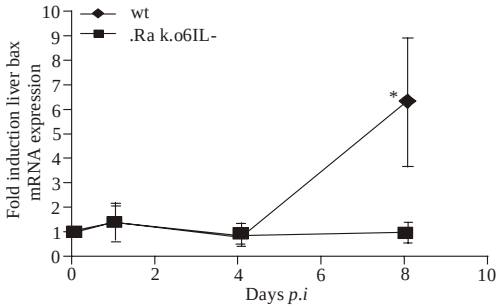

Fig. 3: RT-PCR of Bax gene expression in the spleen of *Plasmodium chabaudi* infected IL-6Rα$^{+/+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5). *: significant value at (p<0.05)

and knockout mice. In the liver of wild type (IL-6Rα$^{+/+}$) mice the level of mRNA expression for Bax as a pro-apoptotic gene was significantly (p<0.05) increased at days 1 and 8 whereas significantly decreased (p<0.05) only at days 1 compared with day 0 (Fig. 2). In contrast, in the Interleukine-6α knockout (IL-6Rα$^{-/-}$) mice the levels of spleen Bax were significantly increased (p<0.05) only at day 8 (Fig. 3). In the IL-6Rα$^{+/+}$ the levels of liver Bcl-2 as an anti-apoptotic gene were significantly (p<0.05) increased at all days after infection (Fig. 4)

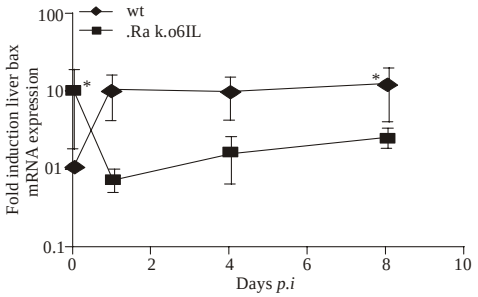

Fig. 4: RT-PCR of Bcl-2 gene expression in the liver of *Plasmodium chabaudi* infected IL-6Rα$^{+/+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5). *: significant value at (p<0.05)

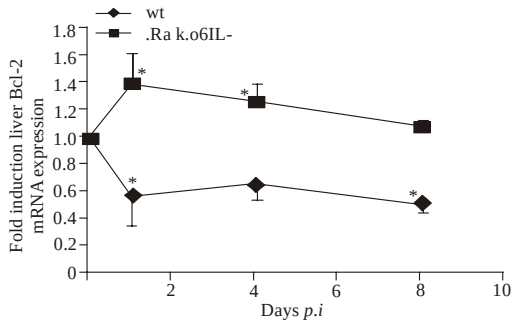

Fig. 5: RT-PCR of Bcl-2 gene expression in the spleen of *Plasmodium chabaudi* infected IL-6Rα$^{+/+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5). *: significant value at (p<0.05)

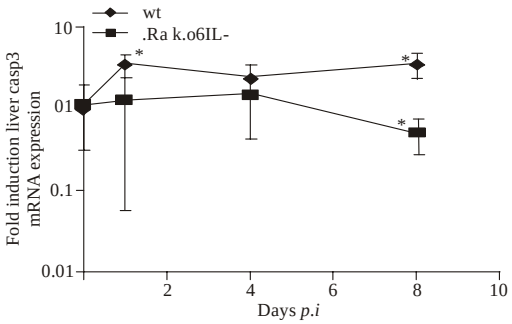

Fig. 6: RT-PCR of Caspase-3 gene expression in the liver of *Plasmodium chabaudi* infected IL-6Rα$^{+/+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5). *: significant value at (p<0.05)

whereas significantly (p<0.05) decreased only at days 1 in the spleen IL-6Rα$^{-/-}$ compared with day 0 (Fig. 5). In the IL-6Rα$^{+/+}$ the levels of liver caspase-3 as an executioner gene were significantly (p<0.05) increased at days 1 and 8 whereas significantly (p<0.05) decreased in the

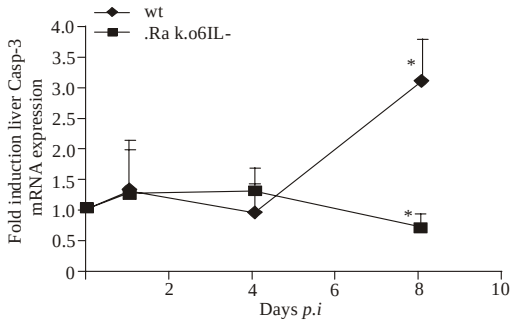

Fig. 7: RT-PCR of Caspase-3 gene expression in the spleen of *Plasmodium chabaudi* infected IL-6Rα^{+}/$^{+}$ mice. The expression of gene was measured at different time points. The data present are the mean±SE (n = 5). *: Significant value at (p<0.05)

IL-6Rα$^{-/-}$ at day 8 compared with day 0 (Fig. 6). However, in the IL-6Rα^{+}/$^{+}$ the levels of spleen caspase-3 were significantly (p<0.05) increased at day 8 only, whereas significantly (p<0.05) decreased at day 8 in the IL-6Rα$^{-/-}$ compared with day 0 (Fig. 7).

DISCUSSION

The major findings of this study were that malaria strongly induced up-regulation of apoptotic genes in the wild type mice by assessing mRNA expression of three measured genes involved in apoptosis (Bax, Bcl-2 and Caspase-3) at different time points. In contrast, these genes were down-regulated in the IL-6Rα$^{-/-}$.

Apoptosis is a complex cellular process whereby cells commit suicide in response to a wide variety of stimuli (Bergmann-Leitner *et al*., 2009). Although apoptosis was originally thought to be an entirely distinct mechanism of cell death, several studies have shown that the processes are regulated by many of the same biochemical intermediates, including death factors and reactive oxygen species (Sanchez-Alonso *et al*., 2004). The data presented here connect with previous results which show that malaria infection significantly induces liver apoptosis mediated by oxidative stress mechanisms and cytokine level change (Guha *et al*., 2006b; Klotz and Frevert, 2008). The pathogen of malaria, Plasmodium, enters erythrocytes and thus escapes recognition by the immune system and then induces oxidative stress to the host erythrocytes and triggers eryotosis (Alkahtani, 2009; Alkahtani *et al*., 2009; Föller *et al*., 2009). Cytotoxins and Reactive Oxygen Species (ROS) generation can increase in many pathological situations and cause cell death (Alkahtani, 2011). Apoptosis is regulated by two major pathways: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. Both pathways converge at the level of active effector caspases. Caspase-independent mechanisms may, however, also contribute to cell death. Effector caspases cleave various cellular target proteins finally leading to apoptosis. In the liver, apoptosis could result from a combination of both pathways: the intrinsic apoptosis pathway by generation of oxidative stress and the extrinsic apoptosis pathway by activation of Kupffer cells which can secrete TNF (Alkahtani, 2009).

The mitochondrial apoptotic pathway plays a critical role in liver cell death during malaria infection (Guha *et al*., 2006b). The intrinsic apoptotic pathway is initiated when Bcl-2 Homology 3 (BH3) domain proteins (the called-so BH3-only proteins) sense cellular stress or developmental cues and lead to the release of cytochrome *c* and other pro-apoptotic cofactors from the inter-membrane space of mitochondria which is considered to be an essential cofactor required for the activation of caspases and is regulated by the Bcl-2 family of proteins upstream of caspase activation (Baliga and Kumar, 2007). Once in the cytosol, cytochrome *c* interacts with the apoptotic protease activating factor-1 (Apaf-1) and the procaspase-9 forming the apoptosome complex (Yuan and Yankner 2000). The result is the cleavage and activation of procaspase-9 and other procaspases that are responsible for the executive stages of apoptotic cell death. The Bcl-2 family of proteins localizes at membrane compartments during apoptosis and can either promote or inhibit apoptosis. Substantial evidence suggests that the primary role of Bcl-2 family proteins is to regulate the release of cytochrome *c* from mitochondria. Briefly, Bcl-2 is an apoptosis suppressing factor that heterodimerizes with Bax and neutralizes the effects of the latter. When Bcl-2 is present in excess, cells are protected against apoptosis. In contrast, when Bax is in excess and the homodimers of Bax dominate, cells are susceptible to apoptosis. Therefore, it is the ratio of Bax to Bcl-2 which determines the fate of a cell (Sanchez-Alonso *et al*., 2004). Infections with intracellular pathogens may provide an appropriate stress-related signal which would normally trigger the intrinsic pathway of apoptosis and, thereby disturb or even prevent the further development of the affected host cells as a response to intracellular pathogens (Graumann *et al*., 2009). The results also demonstrated that the protein levels of Bax as pro-apoptotic molecules decreased after infection compared with control, indicating that an apoptotic cell can eventually undergo secondary necrosis and rupture its contents into the surrounding medium because of the lack of scavenging cells and thus the phagocytic step after apoptosis may not occur (Sanchez-Alonso *et al*., 2004). In addition, Pro-(Bax, Bid, Bad) and anti-apoptotic Bcl-2 members (Bcl-2, Bcl-X_L) regulate the mitochondrial pathway. Since the anti-apoptotic Bcl-2/Bcl-X_L proteins are localized to the outer mitochondrial membrane, they work to prevent Cyt

c release from mitochondria. Recently, it has been reported that the p18/Bax fragment cleaved from full-length Bax (21-kDa) is as efficient as full-length Bax in promoting Cyt *c* release (Oh *et al*., 2004).

Due to their critical functions during apoptosis, Bcl-2 proteins are deregulated by several protozoa which manipulate host cell apoptosis. It is important to note however, that this does not necessarily warrant the conclusion that the parasite-induced up-regulation of anti-apoptotic Bcl-2 proteins is instrumental in protecting the infected host cells from apoptosis. Up-regulation of Bcl-2 proteins following infection can be achieved indirectly via parasite-induced secretion of host growth factors that support the survival of infected cells or both infected and also non-infected bystander cells. Previous studies on Bax mRNA expression levels did show a higher expression in *in vitro* bovine embryos in comparison with co-cultured or *in vivo* cultured embryos. Furthermore, gene expression analysis using RT-PCR indicates the significant up-regulation of Bax expression in the liver of malaria infected mice, suggesting the involvement of mitochondrial pathway of apoptosis (Guha *et al*., 2006b). However, besides the fact that different apoptotic markers cannot always be detected at the same time, very little is known about the exact timing of the successive steps in the apoptotic pathways and for this reason it is suitable to confirm this at the protein level. This is in agreement with the opinion of more and more researchers who argue that the detection of mRNA differences can only indicate a biological significance if protein expression and activity can confirm the results. This is certainly true for caspases because they are secreted as inactive procaspases, which are only active after further modification (Huert *et al*., 2007; Vandaele *et al*., 2008). On the other hand, evidence from several clinical studies indicates that pro-and anti-inflammatory cytokines play a major role in the pathogenesis of malaria. Also, a complex network of inflammatory cytokines and chemokines plays a major role in mediating, amplifying and perpetuating the injury process. Simultaneous production of anti-inflammatory cytokines can potentially counteract pro-inflammatory cytokine effects and modify the intensity of the inflammatory process (Goodman *et al*., 2003; Yang *et al*., 2005).

Concentration ratios of pro-and anti-inflammatory molecular pairs in Bronchoalveolar Lavage Fluids (BALF) provide insight into the inflammatory balance in individual patients and measurement of the net inflammatory activity specific to each cytokine in cellular assays is a key step in validating these calculated ratios. This complexity makes mathematical predictions of the net biological activity difficult when individual cytokine values are compared and underscores the importance of directly measuring net biological activity (Goodman *et al*., 2003).

Supporting these observations, several investigators have recently characterized the biological activity of IL-1β in ARDS edema fluids using microarray analysis. These clinical studies indicate that the interplay between IL-1β and its family of counter-regulatory ligands and receptors probably have an important role in the early pathogenesis of ARDS. Similar to IL-1β, TNF-α is also an important pro-inflammatory cytokine that can stimulate production of a host of other cytokines (Goodman *et al*., 2003). However, Plasmodium sporozoite contact does not elicit a respiratory burst in the hepatic macrophages and blocks the formation of ROS in response to secondary stimuli via elevation of the intracellular cAMP concentration. Stimulation of Plasmodium yoelii sporozoite-exposed Kupffer cells with lipopolysaccharide or IFN-gamma reveals down-modulation of TNF-alpha, IL-6 and MCP-1 and up-regulation of IL-10. Prerequisite for this shift of the cytokine profile are parasite viability and contact with Kupffer cells, but not invasion (Klotz and Frevert, 2008).

However, Kupffer cells are central players in the regulation of the cytokine network of the liver during homeostasis and infection and also serve as a significant systemic source for TNF-α, IL-6, IL-1 and IL-10. However, *in vitro* data indicate that Plasmodium possesses mechanisms to render Kupffer cells insensitive to pro-inflammatory stimuli and eventually eliminates these macrophages by forcing them into apoptosis. In contrast, by manipulating Kupffer cells, *Plasmodium* may be able to support its survival in the host (Klotz and Frevert, 2008). As observed in this study overexpression of apoptotic genes leads to apoptosis. Up-regulation of Bax and/or down-regulation of Bcl-2 mRNA or protein levels has been observed in several experimental models including transient global ischemia, indicating that the expression of Bax and Bcl-2 genes may be regulated by p53 (Mishra *et al*., 2006). More study is also required to elucidate how apoptosis relates to the observed cytokine changes and a better understanding of the underlying mechanisms may take us one-step closer to controlling this deadly parasite.

CONCLUSION

The obtained data revealed that malaria infection with *Plasmodium chabaudi* induces apoptosis in the liver and spleen cells in the IL-6Rα$^{+/+}$ mice via Bax, Bcl-2 and Caspase-3 tests whereas these genes were down-regulated in the IL-6Rα$^{-/-}$. This finding illustrated that the absence of IL-6Rα plays an important role in decreasing the severity of apoptosis during malarial disease. Thus, this study suggests that after further investigation the role of IL-6Rα could be the therapeutic key for determining the outcome of malaria in liver cells. However, because of the apoptosis, detection tests may reveal different results due

to the fact that some cellular protein targets are not affected by caspases. This study suggests confirming the apoptosis test by studying the morphological changes and ultrastructure of cells that may undergo apoptosis.

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