# Interleukin-6, TNF-alpha and interleukin-1 beta levels in blood and tissue in severely burned rats

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Accepted for publication December 22, 2007

**ABSTRACT. Previous studies have demonstrated the early appearance of inflammatory cytokines in the systemic circulation after thermal injury both in humans and animals. The aim of this study was to evaluate the time course of several cytokines, IL-6, TNF-α and IL-1β in serum, lung, liver and brain of severely burned rats during the first week after thermal injury. Cytokine measurements were performed by enzyme-linked immunosorbent assay (ELISA). The comparison between the sham-burned animals and animals with third-degree burns on 20% or 40% of their total body surface area allowed for the study of the inflammatory process relative to the size of the injury. Serum IL-6 levels, which were undetectable in sham-treated animals, peaked during the first hours after injury and were proportionate to the size of the area burned. After a few days, IL-6 increased once more, but only in the most severely burned rats. In lung, liver and brain, low but measurable basal levels of TNF-α and IL-1 were detected in sham-burned animals. Strikingly, IL-1β levels remained significantly elevated in the lung after injury in animals having 20% and 40% burned skin area. Unexpectedly, both TNF-α and IL-1β production decreased gradually in liver and brain after burn injury. Also, the inflammatory response after a burn injury appeared to be biphasic. The first period corresponded to the early release of IL-6 into the circulation, proportional to the severity of the injury. After a few days, a second period was marked by the extension of the inflammatory processes from the injured area to the rest of the body, particularly to lung, which could be considered as at potential risk of involvement in severely burned patients.**

**Keywords:** burn injury, cytokines, interleukins, inflammation

Cytokines are regarded as important mediators in the post-burn pathophysiological process. Notably, the cytokine cascade appears intricately associated with burn-induced oxidative stress [1]. Furthermore, it has been shown that burn injury is associated with reactive oxygen species (ROS) release and lipid peroxidation, which causes oxidative damage to cellular membranes and ultimately leads to cell death [2]. In experimental and clinical studies, burn-associated oxidative stress has been shown by down-regulation of antioxidant activity and oxidation of proteins and lipids in multiple organs [3, 4].

Several studies have suggested that oxidative stress initiates an inflammatory cascade that includes acute phase protein synthesis, upregulation of inflammatory adhesion molecules and proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), particularly in burn injury [1, 5]. However, an activated inflammatory cascade causes a local systemic inflammatory leukocyte influx, which is a source of ROS, and subsequently contributes to the deleterious effects of burn injury.

TNF-α is a potent mediator of the shock-like state associated with thermal injury and sepsis that induces a cascade of secondary cytokines and humoral factors such as interleukin-6 (IL-6) or interleukin-1 (IL-1) [6, 7]. IL-6 is a key cytokine in B-lymphocyte maturation, acute-phase protein induction, and T-lymphocyte regulation, and has consistently been shown to increase after thermal injury [8]. A previous study showed that up-regulation of cytokines (IL-6) correlates proportionally with the extent of the burn injury [9]. IL-1 is a pleiotropic cytokine that regulates the inflammatory response by acting as pyrogen and inducing chemotaxis, maturation and activation of neutrophils and T and B lymphocytes. Although it has been suggested that systemic levels of IL-1 are not increased [10], it could be alternatively implicated in the tissue response after thermal injury.

Given the consequence of oxidative stress in thermal injury and the importance of sustainment of the anti-oxidant enzymatic systems during the medical care of burn victims [11, 12], we studied the time course of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in the complex responses of injured organisms to this severe stressor. We also investigated the levels of these cytokines in serum and various organs, based on their essential role in the systemic

doi: 10.1684/ecn.2008.0113

inflammatory response in burn victims. We took into account the liver, based on the acute phase proteins which are rapidly synthesized during stressful circumstances, as well as the lung which plays an essential role in the systemic circulatory responses in burn victims. Finally, we examined the central nervous system (CNS) response in view of the vital relationship between the CNS and inflammatory processes, which can lead to complicated, additional clinical outcomes such as multiple organ failure syndrome after severe burn injury [13, 14]. Furthermore, it should be noted that the experimental approach used in this study complements the clinical picture observed in MOF syndrome, which generally initiates in the lungs, progresses to the liver, and is followed by involvement of the intestine and kidneys [15].

## METHODS

### *Animal care*

This study was performed in accordance with the French ethical guidelines in order to minimize pain for the animals. Animal housing and experimental procedures were approved by the Animal Ethics Committee of the French Defense Medical Research Center.

Male Wistar rats weighing approximately 250 g were purchased from Charles River Laboratories (France). Prior to being studied, the animals were acclimatized to the local conditions for one week and handled daily to minimize experimental stressors. The animals were housed according to the Agriculture and Environment Ministry standards, which are 21 ± 1°C, 55% relative humidity, and a 12 h/12 h light-dark schedule. They were placed in individual cages and received food and water *ad libitum*. To avoid risk of infection after burn injury, the food, water and litter were sterilized before distribution. A pressurized filter air barrier was established and access to the experimental area was restricted.

### *Design of the study*

The rats were divided randomly into two series of 35 animals; each series was further divided into 7 groups of 5 rats: sham-burned animals (T0) used as control, animals sampled 6 hours after the burn injury (H6) or daily from the first to the fifth day after the burn injury (D1 to D5). The first series (n = 35) underwent a full-thickness burn injury on about 20% of the total body surface area (20% TBSA) (groups $T0_{20}$, $H6_{20}$ and $D1_{20}$ to $D5_{20}$), and the second group (n = 35) on approximately 40% of the TBSA (groups $T0_{40}$, $H6_{40}$ and $D1_{40}$ to $D5_{40}$).

### *Experimental thermal injury*

The 20% or 40% TBSA full-thickness dorsal scald was performed according to the method described by Walker and Mason [16]. Briefly, each rat was anesthetized with halothane, and all dorsal and abdominal hair was clipped. For the 20% TBSA burned rats, the exposed area was delimited in accordance with the body weight of the animals and then the clipped skin of the dorsum was immersed for ten seconds in a boiling water bath (100°C). To stop the burn process, the rats were then immediately plunged into fresh water before awakening. For the 40% TBSA groups, the burned surface corresponded to the entire trunk of the animals. The third degree burn was performed by immersing in 100°C boiled water first for ten seconds the back of the rat, then for five seconds the thinner belly skin of the animal. In the control groups (T0), the animals underwent the same anesthesia as the others, but they were immersed in a room temperature water bath (21°C). After the burn or the sham-burn, all animals were resuscitated by an intra-peritoneal injection of 5 mL of lactated Ringer's solution just after the burn or sham-burn procedure, six hours later, and then daily until sacrifice.

### *Sample collection*

At the end of the experimental period, blood was collected after laparotomy, under halothane anesthesia, by vena cava puncture using Monovette™ syringes (Sarstedt, Germany), without anticoagulant for cytokine dosages. Just after blood collection, the rats were killed by section of heart vessels. The liver, lung and the central nervous system (CNS) were removed and immediately frozen in liquid nitrogen. The serum and the organs were stored at -80°C before analysis.

### *Tissue extract preparation*

After defrosting, 100 mg of liver, the entire lung or the entire CNS were homogenized in ice with a Teflon Potter homogenizer, at a concentration of 1 $mg.mL^{-1}$ in Tris buffer (10 mM DTPA, 10 mM Tris base, pH 7.4) containing protease inhibitors as one tablet for 50 mL of Complete™ and Pepstatin 1 μM (Boehringer Mannheim, Mannheim, Germany), and centrifuged for 10 min, 3 000 g, at 4°C. Then, the supernatant was collected, aliquoted and stored at -80°C for cytokine evaluations. For total protein determination, using a bicinchoninic acid protein assay (BCA; Pierce Chemical, Interchim, Montluçon, France), supernatant was diluted with 1N NaOH (1:1) for 2 hours at room temperature, then centrifuged for 20 min, 12 000 g at 4°C. The supernatant was aliquoted and stored at -20°C.

### *Cytokine assays*

The cytokine (TNF-α, IL-6, IL-1β) levels were determined by specific enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (R&D Systems Europe, France). The minimum detectable doses were less than 5 $pg.mL^{-1}$ for TNF-α and IL-1β, and less than 20 $pg.mL^{-1}$ for IL-6. The serum samples were incubated without dilution. In comparison with a standard cytokine curve, the concentrations of cytokines in serum or in tissue extracts were determined spectrophotometrically (Bio-Tek EL 808, Bio-Tek Instruments Inc, Colmar, France) by reading the absorbance at 450 nm. Cytokine levels were expressed as entire amounts in the serum ($pg.mL^{-1}$), the lung and the CNS (pg), and as relative to total protein levels ($pg.g^{-1}$) in the liver.

### *Statistical analysis*

All data were expressed as mean ± SD. The statistical analysis was performed using Sigma Stat software (SPSS Science, NC, USA). The global course of each parameter

was evaluated by an ANOVA ranks test (Kruskal and Wallis test), and then the comparisons between sham-burn and burn groups by non-parametric rank sum tests (Mann and Whitney tests). On each day, the comparison between the 20% and 40% TBSA rats was performed using a non-parametric rank sum test. The p values less than 0.05 were considered statistically significant.

## RESULTS

### *IL-6*

After burn injury, the progression of IL-6 levels during the first five days were statistically significant regardless of the surface area burned ($p < 0.001$).Within the first six hours after burn injury, IL-6 blood levels increased significantly and peaked the day after the injury. Elevated IL-6 blood levels corresponded to the increased area burned *(figure 1)*. For the rats burned on only 20% of TBSA, transient increases in IL-6 were observed at day 1 (66 $pg.mL^{-1}$, $p = 0.008$) and a plateau occurred within two days following the burn. In comparison, IL-6 blood levels remained elevated in the 40% TBSA group throughout the study. After a early peak of 403 $pg.mL^{-1}$ on day 1 ($p = 0.008$), we observed a second increase in blood IL-6 levels, which progressed from day 3 to reach a subsequent peak of 283 $pg.mL^{-1}$ on day 5 ($p = 0.008$). In the 20% TBSA-burned rats, the second increase in IL-6 levels was less noteworthy with only significant values of ~ 100 $pg.mL^{-1}$ after 8 days (data not shown).

Across groups (20% TBSA- or 40% TBSA-burned and sham-burned rats), we did not observe any significant alterations of IL-6 levels in any organs during the first five days after burn injury. However, IL-6 levels fluctuated in the liver (24 and 148 $pg.mg^{-1}$), CNS (27 and 148 $pg.mg^{-1}$), and lungs (0 and 625 $pg.mg^{-1}$).

### *TNF-α*

TNF-α remained undetectable in all biological samples taken from the 40% TBSA-burned rats. For the rats burned on 20% of TBSA, TNF-α was not detected in serum or lung tissue samples. After 20% TBSA injury, the total amount of TNF-α in the brain decreased progressively from ~ 323 pg to < 75 pg after five days post-burn ($p = 0.036$) *(figure 2)*. In the liver *(figure 3)*, TNF-α levels decreased slightly within the first two days, from 432 to 249 $pg.g^{-1}$ ($p = 0.032$).

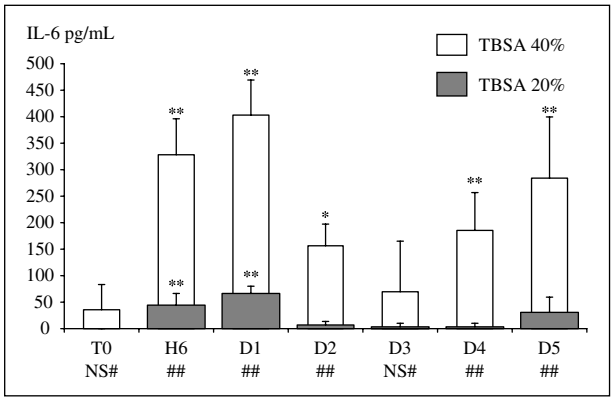


**Figure 1**
Serum IL-6 evolution after burn injury as a function of the injury surface. Data represent mean values (n = 5) ± SD. Difference compared to the sham group: statistically significant at $p < 0.05$ (*) or at $p < 0.01$ (**). Difference compared to the TBSA 20% and 40% TBSA-burned rats: statistically significant at $p < 0.01$ (##) or no statistical difference (NS#).

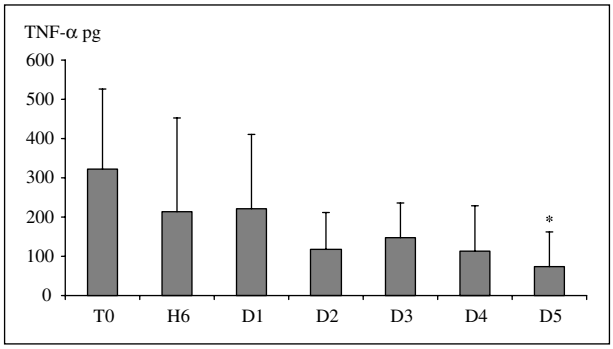


**Figure 2**
Total TNF-α in the CNS of 20% TBSA-burned rats. Data represent mean values (n = 5) ± SD. Difference compared to the sham group: statistically significant at $p < 0.05$ (*).

### *IL-1β*

IL-1β in the serum remained below the levels of detection (31 $pg.mL^{-1}$) in all samples. In the CNS, the total amount of IL-1β decreased rapidly from 679 to 78 pg, a tenth of the initial value, within six hours in the 20% TBSA-burned animals *(figure 4)*. As the area of skin burned increased, the IL-1β values measured in the CNS were ten times lower than those of the 20% TBSA-burned rats. However, there was no statistical difference between the initial levels because of significant individual variability. Nevertheless, in the 40% TBSA rats, IL-1β levels in the CNS decreased very quickly, and then remained reduced for several days *(figure 4)*. In comparison, IL-1β level increased in the lung after burn injury, independently of the burn surface area *(figure 5)*. Significant increases in total IL-1β levels appeared during the first hour after the burn injury (16 774 pg) and peaked the following day (18 243 pg), $p = 0.008$. In both 20% and 40% TBSA-burned rats, the values were twice the initial values. In the lung, IL-1β levels remained elevated over the five days in both 20% and 40% TBSA rats. Conversely, IL-1β levels in the liver were not significant during the first days after the burn

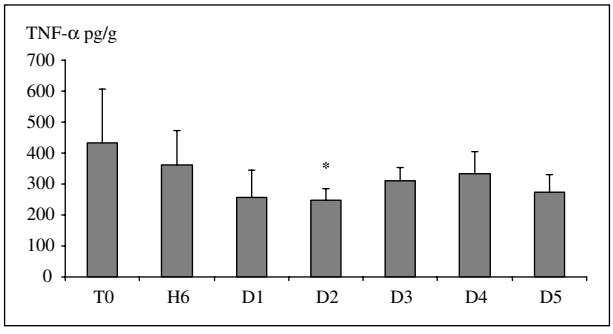


**Figure 3**
Evolution of TNF-α levels in liver in 20% TBSA-burned rats. Data represent mean values (n = 5) ± SD. Difference compared to the sham group: statistically significant at $p < 0.05$ (*).

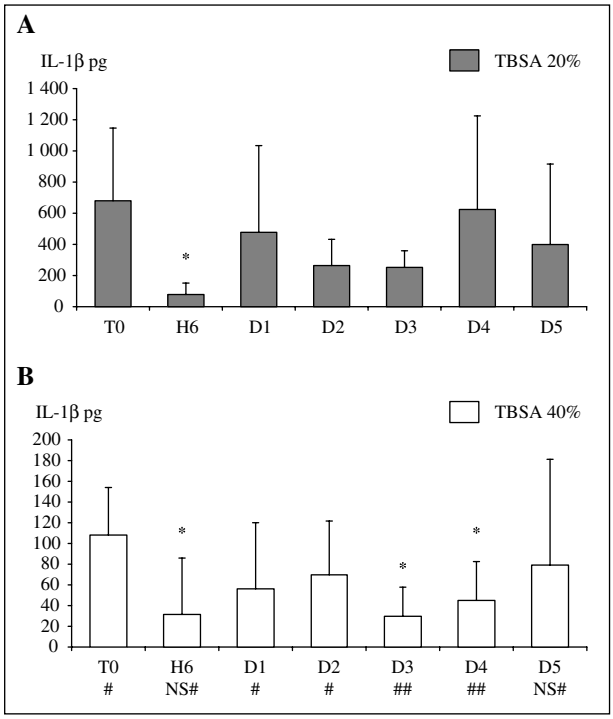


**Figure 4**
Total IL-1β evolution in CNS after burn injury as a function of the injury surface, in 20% TBSA- (**A**) and 40% TBSA- (**B**) burned rats. Data represent mean values (n = 5) ± SD. Differences compared to the sham group (T0): statistically significant at $p < 0.05$ (*). Statistically significant differences between the 20% TBSA- and 40% TBSA-burned rats: at $p < 0.05$ (#), at $p < 0.01$ (##) or no statistical difference (NS#).

injury, 3 000 to 4 000 pg.g$^{-1}$ *(figure 6)*. In the 40% TBSA rats, IL-1β increased by day 4 and was twice the initial value (6 248 pg.g$^{-1}$) by day 5 ($p = 0.016$).

## DISCUSSION

In this study, we investigated the time course of proinflammatory cytokines in a severely burned rat model. In animals with 20% or 40% of their TBSA burned, the skin injury was well tolerated throughout the seven days following the burn. We acknowledge that in rodents the clinical picture is quite different from humans, in that skin lesions are not as exudative and have fewer pathophysiological consequences. However, our model allowed us to study the cascade of events that results in burn-induced inflammation and the influence of the surface area of the burn on these factors.

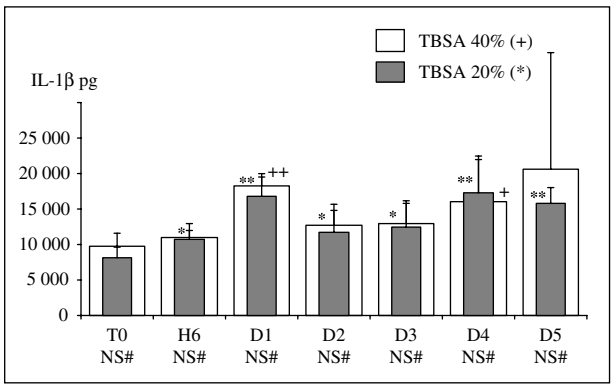


**Figure 5**
Comparative evolution of total IL-1β in the lung from 20% or 40% TBSA-burned rats. Data represent mean values (n = 5) ± SD. Differences compared to the sham group (T0): statistically significant at $p < 0.05$ (*) or $p < 0.01$ (**). No statistically significant difference between the 20% TBSA- and 40% TBSA-burned rats was noted at any time (NS#).

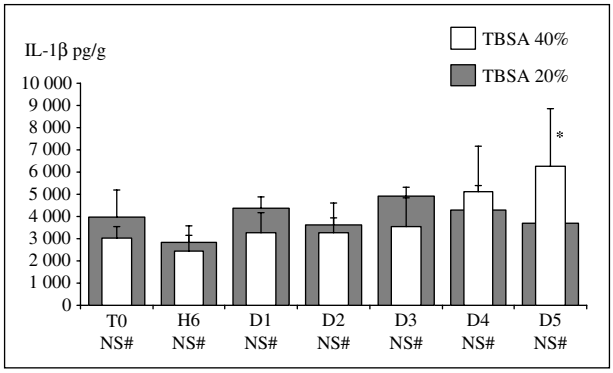


**Figure 6**
Evolution of IL-1β levels in liver after burn injury as a function of the injury surface. Data represent mean values (n = 5) ± SD. Difference compared to the sham group (T0): statistically significant at $p < 0.05$ (*). No statistically significant difference between the 20% TBSA- and 40% TBSA-burned rats was noted at any time (NS#).

Immediately following the injury, a proinflammatory cytokine release has been described both in burn patients and in animal models. In humans, the presence of cytokines in the plasma of healthy volunteers remains undetectable; however, IL-6, IL-1β, TNF-α, IL-2, IL-10, IL-13, IL-8 and G-CSF emerge as the main cytokines released into plasma during the first few days after a burn injury [17-19], which are related primarily to the synthesis of acute phase proteins and dissemination of the inflammatory response. In addition, dysregulation of the immune response increases the risk of superinfection. Conversely, other cytokines such as IL-4 or GM-CSF remain unchanged [17]. Nevertheless, rodent models do not precisely follow human clinical observations and the infrequent, published findings are contradictory. In mice, plasma levels of IL-6, TNF-α and IL-10 are not elevated from 1 to 24 hours following a 20% TBSA burn [20]. Nevertheless, blood samples of 20% TBSA-scalded rats show significant increases in IL-6 and TNF-α at days 3 and 7 post-burn [21]. In 30% TBSA-burned rats, TNF-α and IFNγ peaks occur within 3 hours after injury [22]. Caldwell showed a precocious release of IL-6 in the plasma of 50% TBSA rats, with a delayed increase in IL-1β during the first week after injury [23]. Recently, in 60-70% TBSA-burned rats, significant increases in TNF-α and IL-1β protein levels were observed seven hours after burn injury [24]. In our study, we did not observe any changes in IL-1β or TNFα levels in serum during the first days after 20% or 40% TBSA burn injuries. We observed a precocious release of IL-6 six hours following, and the first day after injury, and levels were proportionate to the size of the burned area as previously described [25, 26]. After this initial peak, serum IL-6 levels in 40% TBSA-burned rats remained significantly higher than in control rats and less burned rats, and had a tendency to increase progressively throughout the first five days. Tissue release of circulating IL-6 is remains a possibility; however, our data do not show detectable IL-6 levels

in liver, kidneys or lungs in animals over the five days following the burn. Previous studies have suggested a potential role of the unburned skin surrounding the lesions; thus, it appears that underlying muscle is less reactive during the first 24 hours after the scald wound [20]. During the second phase, release of pro-inflammatory IL-6 in the systemic circulation probably corresponds to the activation of inflammatory cell populations or endothelial cells, and contributes to the extension of the inflammatory response from initially injured local areas to the entire organism in the more severe cases. As after thermal injury, IL-6 is well known to modify the cell-mediated immune response, particularly in terms of delayed-type hypersensitivity and splenocyte proliferative response [27], and therefore plays a significant role in the pathophysiology of sepsis in burn patients [28-30]. It appears particularly interesting to take into account the consequences of IL-6 release on the different cell populations in the different organs in burned rats. Because of its intrinsic properties, IL-6 may upregulate or suppress local cytokine mRNA synthesis and therefore modulate the expression of other proinflammatory cytokines in distant organs.

In the lung, we observed an increased synthesis of IL-1β as early as the sixth hour after the burn injury in both 20% and 40% TBSA groups, regardless of the the extent of the wound. The presence of increased amounts of IL-1β in the pulmonary parenchyma remained statistically significant throughout the five days of the study. IL-1β was not detectable in the blood at any time and the presence of cytokines are consistent with local activation of gene synthesis with a potential effect on circulating factors produced near the initial wound area or by splanchnic nervous system stimulation. These results are consistent with previous observations that IL-1α increases in lung one hour after the burn without any change to blood levels [31]. Although the mechanism of the post-injury cytokine cascade is complex, and there are diverse opinions in the scientific community, IL-6 appears rather as an anti-inflammatory cytokine and its early increase is likely not due to pulmonary synthesis of IL-1β [32, 33]. On another hand, IL-1β gene transcription may be activating several transcriptional activators such as NF-κB or NF-IL-6, depending on the presence of IL-6 [34]. Concurrently, the precocity of the IL-1β increase and the absence of any symptoms of systemic infection during the first days after injury suggest a specific local mechanism, independent of any endotoxemia process induced by digestive bacterial translocation [31]. Piccolo *et al*. demonstrated that the lung expression of IL-1 after burn injury is dependent on chemotactic mediators such as C5a complement fragment, keratinocyte-derived cytokine (KC) or macrophage inflammatory protein-2 (MIP-2) [35]. The effects of such a local release of IL-1β may be particularly deleterious for the lung of burned animals in that proinflammatory cytokines may increase the alveolar permeability to proteins, participate in the alveolar edema, and favor polymorphonuclear infiltration in the lung. The rapid and sustained elevation of IL-1β levels in lung represents a potential risk factor that maintains an unhealthy terrain propitious for pulmonary infections, not to mention the effects of other inflammatory regulators in the inflammatory process [36]. Pulmonary infections are still particularly troublesome in severely burned patients despite recent advances in the medical management [37]. The therapeutic control of proinflammatory conditions in lung may be particularly interesting in order to limit the pulmonary consequences of the burn injury. In this way, the neutralization of inflammatory chemoattractants by specific antibodies against C5a, MIP-2 or KC is effective for reducing the generation of local IL-1 [35], and therefore probably limits the expression of the endothelial adhesion molecules, E-selectin and ICAM-1 and then pulmonary neutrophil recruitment [38]. The control of synthesis of local IL-1β appears to be a better solution to regulate the inflammatory response. Indeed, the fact that IL-1β remains undetectable in the circulation despite its local pulmonary production raises the questions as to whether IL-1β produced is secreted out of cells and upon which target it does it act. This would increase the possibility of inhibiting the action of IL-1β by competition with different antagonists such as IL-1RA.

Over the first 5 days following the burn injury, changes in hepatic levels of the proinflammatory cytokines studied are limited to a depression of TNF-α, with a tendency to increased IL-1β levels in more severely burned rats within four or five days. The progressive decrease in TNF-α level is inconsistent with most of the observations, which describe an amplification of its synthesis in trauma. This is related to the influence of the early release of IL-6 within six hours to the first day after the injury. The downregulation of TNF-α synthesis by IL-6 has been demonstrated by the study of IL-6-deficient, knockout mice which present with markedly elevated TNF-α levels and with *in vitro* culture models [33, 39]. Further studies are needed to establish the present mechanism for the downregulation of TNF-α synthesis. During the second phase, the delayed increase in IL-1β in the liver of 40% TBSA-burned rats appeared to be unrelated to the precocious inflammatory events, and it underlines the progressive evolution of inflammatory process in more severely injured animals, despite the absence of any clinical symptoms. The local synthesis of IL-1 may thus be particularly efficient in amplifying the inflammatory response because of its ability to modulate the expression of the genes coding for liver-derived plasma proteins [40]. This clinical observation has been recently confirmed by *in vitro* studies related to the gene profile induced by IL-1β in liver-derived cell lines [41].

As in the liver, we observed in CNS a tendency for decreased proinflammatory cytokines, such as TNF-α and IL-1β,which is in contrast to previously published data which all describe an upregulation of these cytokines after illness, wound or sepsis [24, 42]. In contrast to in all other experimental injury models we use in the French Defense Medical Research Center, particularly those involving muscular exercise, heat stroke, septic shock or radiological burns, thermal burn injury is the only experimental situation in which we observe a downregulation of TNF-α and IL-1β in different organs, following injury. Further proteomic and genomic research is now ongoing in order to compare the pathophysiological pathways in all cases of injury, and to elucidate the regulatory mechanisms involved in these issues. As the precise mechanisms of cytokine production after *in vivo* brain injury remain unelucidated, we hypothesis that the different cytokine levels seen following burn injury, could be affected by a variety of mechanisms, from regulation of synthesis genes by NF-κB, p38 MAPK or TLR4 activation, to maturation of the pro-IL-1β by caspase-1, for example [43]. In any

experimental situation, such as inhibition of p38 MAPK to protect rats from cerebral ischemia, IL-1β and TNF-α indeed decrease in the brain, emphasizing the importance of such gene regulators in the synthesis of these cytokines [44]. However, as early as 3 hours after thermal injury, a significant increase in TNFα, IL-1β and ICAM-1 mRNA expression is observed in brain from 60-70% TBSA-burned rats, with levels remaining elevated seven hours after injury [24]. Other reports concerning the reduction of the inflammatory response in the CNS of TLR4-knockout mice underline the relevance of the study of this pathway to explain our results [45]. The study of the fine regulation of cytokine synthesis in brain following burn injury merits further attention.

In conclusion, after a skin burn injury to 20% or 40% of the TBSA, changes in inflammatory cytokine levels were observed within the first five days, despite the absence of behavioral disturbances in the injured Wistar rats. As early as the sixth hour following the injury, the organism responded with release of IL-6 into the circulation. We initiated biological sampling of the animals 6 hours after the burn injury; however, it remains possible that release of IL-1β or TNF-α began prior to this time. After these initial responses to the burn injury, we observed secondarily progressive responses which were dependent upon the extent of the burn injury. This period presented a dual inflammatory response depending on the organ studied which might appear, to a certain degree, inconsistent. In the lung, the increase in IL-1β in the absence of any infection, showed the potential for generalization of the inflammatory reaction from the cutaneous, burned areas to other, distant, deep organs, suggesting the risk of systemic inflammatory response syndrome (SIRS) and with the possibility of progression to MOF syndrome in the worst clinical scenarios. The fact that circulating levels of IL-6 gradually increased after a few days, confirms this observation. However, during this period, an unexpected anti-inflammatory response occurred, with a decrease in proinflammatory cytokines such as IL-1β and TNF-α in the CNS and liver, and which was more marked as the burn size increased. In order to design appropriate pharmacotherapy strategies, we need to consider the development of the cytokine cascade in our experimental model. Further studies are warranted to understand precisely the regulation of inflammatory processes following thermal burn injury.

***Acknowledgments***. *The authors thank Robert Carter for his kind assistance in the English revision of the manuscript. This work was supported by a grant from Délégation Générale à l'Armement (DGA, France). The authors have no financial or proprietary interest in the subject matter.*

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