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# Several Functions of Immune Cells in Mice Changed by Oxidative Stress Caused by Endotoxin

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## Summary

We have studied natural killer (NK) activity, lymphoproliferative response, the release of several cytokines (IL-2, TNFα and IL-1β) and the ROS production in peritoneal leukocytes obtained 0, 2, 4, 12 and 24 h after lipopolysaccharide (LPS) injection. Lethal septic shock (100 % mortality occurred at 30 h after LPS administration) was caused in female BALB/c mice by intraperitoneal injection of 100 mg/kg of *E. coli* LPS. Cytotoxicity and lymphoproliferation assay were preformed together with the measurement of IL-1β, IL-2 and TNFα production, and quantification of ROS. Natural killer activity, spontaneous lymphoproliferative response, IL-2, TNFα, IL-β release and ROS production were increased after LPS injection. In conclusions, ROS and proinflammatory mediators produced by immune cells in response to LPS are involved in the oxidative stress of endotoxic shock. This oxidative state alters some functional characteristics of leukocytes (proliferation and NK activity).

**Key words**

Cytotoxic activity • Leukocytes • Proinflammatory mediators • Proliferation

## Introduction

The generation of an immune response to infectious agents involves the activation of effector cells such phagocytes, lymphocytes and natural killer (NK) cells, as well as a subsequent production of cytokines and other mediators, mainly reactive oxygen species (ROS). This ROS production is involved in oxidative stress and subsequent morbidity and mortality linked to an excessive activation of immune cells in infectious processes (Henson and Johnston 1987, Novelli *et al*. 1989, Víctor *et al*. 1998). TNFα is a proinflammatory cytokine greatly involved in the pathophysiological changes associated with acute and chronic inflammatory conditions, including septic shock, autoimmune diseases, wasting, rheumatoid arthritis, inflammatory bowel disease and the respiratory distress syndrome (Beutler and Cerami 1989, Vassalli 1992). Another proinflammatory cytokine, namely the IL-1β, has also been involved in the pathology of endotoxic shock (Kuwagata *et al*. 2000). Although the release of ROS is stimulated by several cytokines, including TNFα and IL-1β (Jensen *et al*. 1992), the oxygen intermediates, in turn, up-regulate cytokine synthesis by leukocytes, creating a vicious circle for the production of both types of inflammatory mediators (Wendel 1991, Pogrebniak *et al*. 1992). Immune cells are particularly sensitive to oxidative stress because of their higher production of ROS which play a role in their normal function (Meydani *et al*. 1995) as important signaling molecules in the regulation of various cellular processes (Kim *et al*. 2001). Thus, the oxidant-antioxidant balance is critical for



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immune cell function (Knight 2000). The NK activity, that is very important in the innate immune response to infection and tumors (Trinchieri 1987), is carried out through production of ROS (Preston *et al.* 2001). The proliferation of lymphocytes is a pivotal function of the immune response triggered by antigens or mitogens (Globerson 1995). However, an excessive spontaneous lymphoproliferation can be a risk for the health of individuals (Smith 2001). In this proliferation, ROS are also involved (Kim *et al*. 2001), and the proliferation and NK activity are controlled by cytokines such as IL-1β and mainly IL-2 (Hefeneider *et al*. 1983, Siegel *et al*. 1997, De Guise *et al*. 2000).

We have previously observed changes in several functions of peritoneal macrophages from BALB/c mice with lethal septic shock induced by endotoxin (lipopolysaccharide, LPS) injection, especially a decrease of chemotaxis capacity, an increase of adherence, phagocytosis and a very high production of superoxide anions, which represent the first oxygen free radicals in the "respiratory burst" (Víctor *et al*. 1998, Victor and De la Fuente 2003). Although it is well known that phagocytes are involved in endotoxic shock, other immune cells such as lymphocytes also seem to participate in the oxidative stress produced in response to endotoxin (Víctor et al. 2002). Moreover, the oxidative state produced in response to LPS at high concentrations, which results in death of the animal, could induce changes of several immune functions in response to infection. Therefore, the aim of the present work was to study the changes in NK activity, lymphoproliferation (spontaneous and in response to the mitogen concanavalin A), the release of cytokines such as IL-2, IL-1β and TNFα as well as ROS production by the peritoneal leukocytes from female BALB/c mice with a lethal endotoxic shock caused by intraperitoneal injection of 100 mg/kg of LPS *E. coli*.

## Methods

*Animals*

Adult female BALB/c mice (*Mus musculus*) (Harlan Interfauna Ibérica, Barcelona, Spain), were maintained at a constant temperature (22±2 $^{o}$C) in sterile conditions inside an aseptic air negative pressure environmental cabinet (Flufrance, Cachan, France) on a 12 h light/dark cycle and fed Sander Mus pellets (Panlab L.S. Barcelona, Spain) and drinking water *ad libitum*. The animals used did not show any signs of malignancy or other pathological processes. Mice were treated according to the guidelines of the European Community Council Directives 86/6091 EEC. Although we have previously observed that the estrous cycle phase of mice had no effect on this experimental assay, all females used in the present study were in the initial stages of diestrus.

*Experimental protocol*

A group of 8 animals was used. Endotoxic shock was induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (055:B5), at a concentration of 100 mg/kg. Each animal was injected with LPS between 9:00 and 10:00 h.

*Collection of cells*

At 0, 2, 4, 12 and 24 h after LPS injection, peritoneal suspensions were obtained without sacrificing the mice by a procedure previously described (De la Fuente 1985). Briefly, 3 ml of Hank's solution, adjusted to pH 7.4, were injected intraperitoneally, then the abdomen was massaged and the peritoneal exudate cells were collected allowing recovery of 90-95 % of the injected volume containing leukocytes which were identified by morphologic and cytometric assay and counted in Neubauer chambers (Blau Brand, Germany). The cellular suspensions showed a viability of 99±1 %.

*Cytotoxicity assay*

An enzymatic colorimetric assay was used for cytolysis measurements of target cells (Cytotox 96 TM Promega, Boehringer, Ingelheim) based on the determination of lactate dehydrogenase enzyme (LDH) using tetrazolium salts, as previously used by us on similar samples (Ferrandez *et al.* 1999). Murine lymphoma YAC-1 cells were used as the target in the NK assays. The cells were maintained in a complete medium which consisted in RPMI-1640 without phenol red (Gibco, Canada Ltd, Burlington, Ontario) plus 10 % of heat-inactivated (56 $^{o}$C, 30 min) fetal calf serum (Gibco, Canada Ltd, Burlington, Ontario). Target cells were seeded in 96-well U bottom culture plates (Orange Scientific, Belgium) at $10^4$ cells/well. Effector cells, peritoneal leukocytes, adjusted at $10^6$ cells/ml of RPMI-1640 were added at $10^5$ cells/well, with an effector/target rate of 10/1. The plates were centrifuged at 250 x *g* for 4 min to facilitate cell contacts and then they were incubated for 4 h at 37 $^{o}$C. After incubation, LDH activity

was measured in the supernatants (50 µl/well) after addition of the enzyme substrate; the absorbance recording was performed at 490 nm. Three kinds of control measurements were performed: a target spontaneous release (TS), a target maximum release (M), and an effector spontaneous release (ES). To determine the percentage of lysis of target cells, the following equation was used: % lysis = ((E-ES-TS)/(M-ES-TS))x 100, where E = mean of absorbances in the presence of effector cells, ES = mean of absorbances of effector cells incubated alone, TS = mean of absorbances in target cells incubated with medium alone, and M = mean of maximum absorbances after incubating target cells with the lysis solution.

*Lymphoproliferation assay*

A previously described method was used (Del Río *et al.* 1994). Aliquots (200 µl) of leukocytes, adjusted to $1x10^6$ cells/ml of complete medium, were seeded in 96-well flat-bottomed microtiter plates (Orange Scientific, Belgium). Twenty µl of concanavaline A (Con A, 1 mg/ml, Sigma, St Louis, MO) or 20 µl of medium (spontaneous proliferation) per well were added and the plates incubated for 48 h at 37 °C in an atmosphere of 5 % $CO_2$. Thereafter, 0.5 µCi $^3$H-thymidine (Du Pont, Boston, MA, USA)/well was added and after 8 h of incubation the cells were harvested in a semiautomatic microharvester and thymidine uptake was measured in a beta counter (LKB, Uppsala, Sweden) for one minute. The results were expressed as $^3$H-thymidine uptake (cpm).

*IL-2 production assay*

The concentration of interleukin-2 (IL-2) was measured using an ELISA kit (R&D Systems, Minneapolis, USA) in the supernatants of above indicated culture of peritoneal leukocytes. The results are expressed as pg/ml, and the minimum detectable dose and the quantification limit of mouse IL-2 was 3 pg/ml and 1000 pg/ml, respectively.

*TNFα and IL-1β release*

TNFα and IL-1β release were determined in the supernatants of cultures of peritoneal leukocytes incubated with Hank´s solution at a final concentration of $2x10^5$ cells/200 µl/well in 96 well plates for 24 h. The concentration of cytokines was measured using an ELISA kit (Endogen, Woburn USA), and the results for both cytokines were expressed as pg/ml, with a minimum detectable dose of mouse TNFα and IL-1β of 10 and 3 pg/ml, respectively, and a quantification limit of 1500 and 1000 pg/ml, respectively.

*Reactive oxygen species production*

ROS production was measured by flow cytometry using dichlorodihydrofluoresceine diacetate (DDF-DA) as a probe since it is oxidized in the cytoplasm by ROS to 2'7'-dichlorofluoresceine (DCF), which is a highly fluorescent compound. Aliquots of 200 µl of the peritoneal suspension (adjusted to $10^6$ cells/ml) were centrifuged for 10 min at 1500 rpm and 4 °C. The supernatants were discarded and the pellets were resuspended in 200 µl of buffer A (Hank's medium without $Ca^{+2}$ and $Mg^{+2}$ and with EGTA (1 mM). The samples were incubated with 2 µl of DDF-DA (0.5 mM) for 15 min at 37 °C. Then, the samples were incubated for 15 min at 37 °C and, thereafter they were analyzed using a FACScan flow cytometer (Becton Dickinson, San Diego, USA). The results were expressed as fluorescence units (F. U.).

*Statistical analysis*

The data are expressed as mean ± S.D., the number of experiments is shown in the figures. The data were analyzed by one-way repeated measures analysis of variance (ANOVA) since the data did not show a Gaussian distribution for the different groups of mice at 0, 2, 4, 12 and 24 h after LPS injection. The Bonferroni and Student Newman Keuls tests with a level of significance set at $P<0.05$ were used for *post hoc* comparisons.

## Results

The values of NK activity, spontaneous lymphoproliferation and IL-2 of peritoneal leukocytes at 0, 2, 4, 12 and 24 h after LPS injection are shown in Figure 1. These functions were increased with respect to time 0 (control) at all times after LPS injection ($P<0.01$). However, the proliferative response of lymphocytes to the mitogen Con A and the corresponding IL-2 values (Fig. 2) decreased at all times after LPS injection ($P<0.01$).

The results of IL-1β and TNFα release (Fig. 3) show, with respect to time 0 (control), an increase at all times after LPS injection. However, this effect was

significantly higher at 2 and 4 h ($P<0.01$), and at 2 h ($P<0.001$) after LPS injection for IL-1β and TNFα, respectively.

ROS production by peritoneal leukocytes (Fig. 4) is increased at all times after LPS injection, with the peak at 24 h ($P<0.001$).

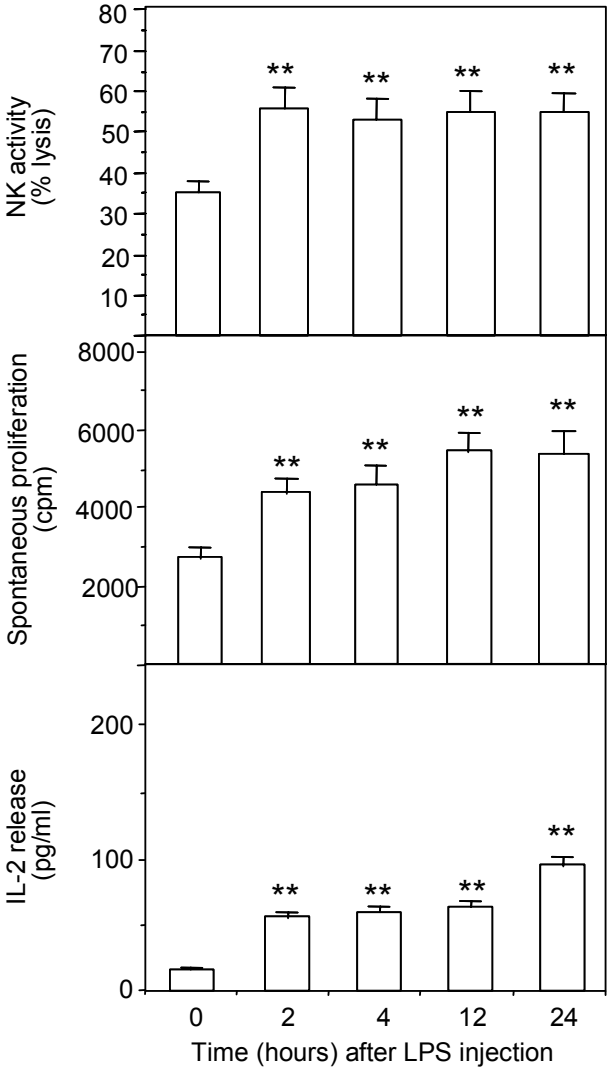


**Fig. 1.** *NK activity (% lysis of tumor cells), as well as spontaneous proliferation (cpm) and IL-2 (pg/ml) release in cultures, in the absence of concavalin A, of peritoneal leukocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection of LPS. Each column represents the mean ± S.D. of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. **p<0.01 with respect to the corresponding values at 0 h.*

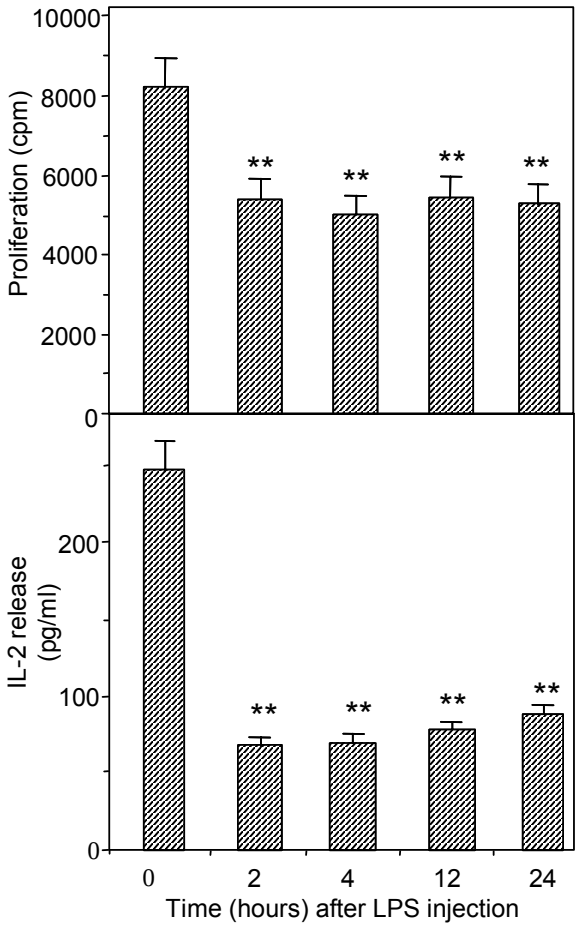


**Fig. 2.** *Proliferation (cpm) and IL-2 release (pg/ml) in cultures, in the presence of concavalin A, of peritoneal leukocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± S.D. of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. **p<0.01 with respect to the corresponding values at 0 h.*

## Discussion

Leukocytes are widely recognized as cells that play a central role in the regulation of inflammatory activities (Laskin and Pendino 1995). Overproduction of proinflammatory cytokines and ROS, which in adequate amounts are involved in normal and localized immune defenses, leads to the development of an oxidative stress which occurs in endotoxic shock (Victor and De la Fuente 2000, 2003). Peritoneal macrophages and lymphocytes play an essential role in the host immune response to inflammatory and infectious processes such as endotoxic shock (Baeuml *et al*. 1997, Victor *et al*. 2000, 2002, Victor and De la Fuente 2000, 2003, De la Fuente and Victor 2001) as producers (especially the former) of inflammation mediators such as TNFα and

ROS. In the present work, we have observed that LPS injection (100 mg/kg) results in a significant increase of ROS production, as well as of IL-1β and TNFα release by peritoneal leukocytes from BALB/c mice, generating an oxidative stress situation. However, as the levels of TNFα and IL-1β depend on the receptor-ligand balance of these cytokines, the evaluation of their receptors would be necessary before ensuring that there is an increase of TNFα and IL-1β release by leukocytes from mice injected with LPS. Oxidative stress was also shown in previous studies through changes of other leukocyte functions such as adherence and chemotaxis of macrophages and lymphocytes or phagocytosis of macrophages(Victor *et al.* 1998, 2002, Victor and De la Fuente 2000, 2003)**.**

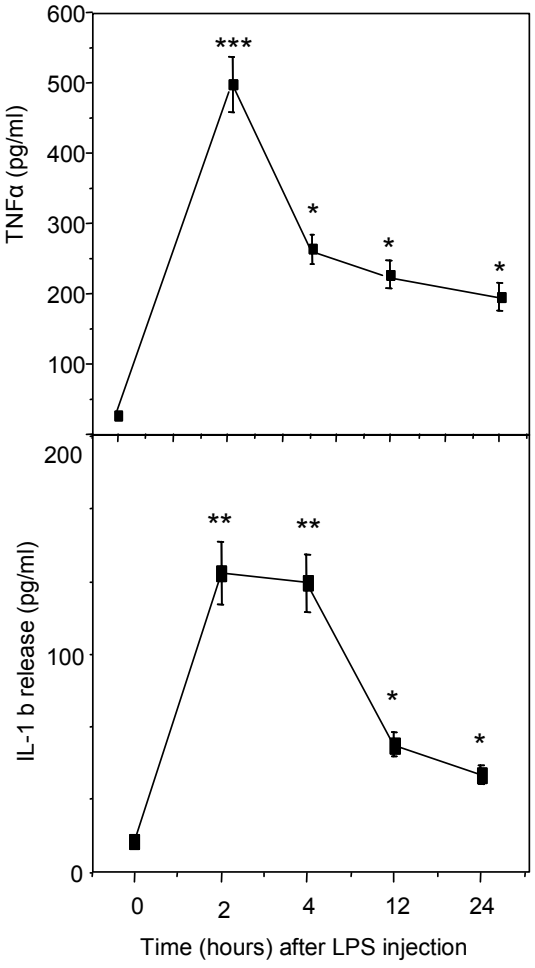


**Fig. 3**. *TNFα and IL-1β release (pg/ml) of peritoneal leukocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± S.D. of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. *p<0.05, **p<0.01 and ***p<0.001 with respect to the corresponding values at 0 h.*

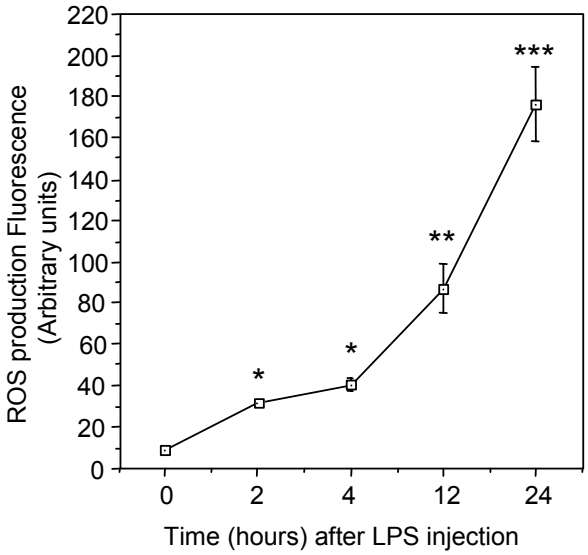


**Fig. 4**. *ROS production (arbitrary units of fluorescence) of peritoneal leukocytes. In all cases, the cells were obtained at 0, 2, 4, 12 and 24 h after injection. Each column represents the mean ± S.D. of 8 values corresponding to 8 animals, each value being the mean of duplicate assays. *p<0.05, **p<0.01 and ***p<0.001 with respect to the corresponding values at 0 h.*

Immune cell functions, such as NK and lymphoproliferative activity, are linked to reactive oxygen species and proinflammatory cytokines (Kim *et al*. 2001). In the present work, we observed that these functions are increased after LPS injection. Endotoxin stimulates the NK activity of peritoneal leukocytes which could be due to the increase of IL-2 or TNFα since both cytokines are involved in the regulation of NK activity (Karahashi and Amano 2000, Goodier and Londei 2000)**.** Moreover, TNFα is implicated in the IL-1β and IL-2 release (Mathew and Sharma 2000). In agreement with the findings of Haller *et al.* (2000), an increment of spontaneous lymphoproliferation activity has been demonstrated at all times after LPS injection in the present work. It is known that IL-1β and IL-2 are implicated in the proliferation of T lymphocytes (Mathew and Sharma 2000), and TNFα is also implicated in the regulation of cell proliferation (Tribouley *et al*. 1999). LPS stimulates the release of these cytokines and it thus increases the lymphoproliferation (Trinchieri 1987, Ceuppens *et al*. 1988, Abuharfeil *et al*. 2001). However, excessive production of IL-1β in response to microbial products, e.g. LPS, could contribute to severe pathological changes and death in both animal and human infections (Rausch *et al*. 1994). In the presence of concavalin A, a mitogen that mimics the stimulation of

lymphocytes by antigens (Sharon 1983), the lympho-proliferative response as well as the IL-2 release decrease at all times after LPS injection. Although LPS acts as a mitogen for B lymphocytes (Frisan *et al*. 2000) and concavalin A for T lymphocytes (Kumar and Chakrabarti 2000), our results show that the peritoneal injection of LPS can induce IL-2 release and consequently T cell proliferation of peritoneal leukocytes. This fact and the subsequent presence *in vitro* of concavalin A could explain the decrease in the IL-2 release and T cell proliferation. In fact, similar results have been observed in cultured cells in the presence of neuropeptides and concavalin A; these neuropeptides stimulate spontaneous proliferation which is inhibited in the presence of concavalin A through a decrease of IL-2 release (De la Fuente 1999). and De la Fuente 2000)**.**

In summary, the above data suggest that peritoneal leukocytes are involved in the pathogenesis of endotoxic shock contributing to the oxidative stress produced by LPS through ROS production and proinflammatory cytokine release. Moreover, this oxidative condition is associated with changes in several immune functions such as NK activity, lymphoproliferation and IL-2 release. These results are important for understanding the deregulated immunity that takes place in the leukocyte response to lethal endotoxic shock as a consequence of the oxidative stress situation.

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