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Consequences of the lack of IL-10 in different endotoxin effects and its relationship with glucocorticoids

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Running head: Relationship between IL-10 and GC in an endotoxin context.

ABSTRACT

Sepsis constitutes one of the major causes of death in intensive care units. In sepsis induced by Gram-negative, while LPS initially induces an exacerbated secretion of proinflammatory cytokines leading to endotoxic shock and death resembling a septic shock, it is also capable of inducing refractoriness to subsequent challenge with LPS, a state known as endotoxin tolerance, which is considered the initial step of the immunosuppression found in septic patients. Since we previously demonstrated the importance of glucocorticoids in endotoxin tolerance, the aim of this study was to evaluate the contribution of IL-10 both in the endotoxic–shock and in the development of the tolerance and its relationship with glucocorticoids. Our results show that, upon LPS challenge, IL-10 KO mice had an enhanced LPS sensitivity, along with elevated levels of proinflammatory cytokines as TNF-α, IL-12 and IFN-γ, and enhanced tissue damage, despite the high levels of glucocorticoids. This effect may be due, in part, to the higher expression of TNFRs in IL-10 KO mice. Further, the injection of dexamethasone did not protect IL-10 KO mice from a LPS lethal challenge. While tolerance was achieved in the absence of IL-10, it was weaker and the elevated levels of glucocorticoids were not able to reverse the high sensitivity of IL-10 KO mice to LPS. Nevertheless, glucocorticoids would play a pivotal role in the establishment and maintenance of this partial tolerance in IL-10KO mice. Finally, our results show that IL-10 and glucocorticoids could act in a bidirectional way influencing the inflammatory and anti-inflammatory periods.

Keywords: LPS; endotoxin tolerance; sepsis; IL-10; glucocorticoids

ABBREVIATIONS:

WT, Wild-type mice

IL-10 KO, IL-10 knockout mice

LPS, lipopolysaccharide

i.p., intraperitoneally

s.c., subcutaneously

TOL, tolerant

AST, aspartate transaminase

ALT, Alanine aminotransferase

Cre, creatinine

MFI, mean of fluorescence intensity

GC, glucocorticoids

DEX, dexamethasone

PBS, Phosphate-buffered saline

INTRODUCTION

Sepsis is one of the leading causes of death in patients in intensive care units. The global incidence of this syndrome has been gradually increasing as a consequence of longevity in the population and the increase of drug-resistant pathogens (1, 2). For these reasons, the World Health Organization recently recommended to reinforce preventive measures and research of new diagnostic methods and treatment (3).

In the last 40 years and after more than 150 clinical trials, sepsis therapies focused on the attenuation of the initial systemic inflammatory syndrome response (SIRS) have shown only modest benefits (2). One important reason of this failure was the little attention paid to the development of compensatory anti-inflammatory response syndrome (CARS), a dynamic period that, frequently, concludes in a severe immunosuppression and where the majority of deaths occur (4, 5). The resolution of inflammation, as well as the induction of immunosuppression involves intricate networks of effector cells and molecules, among which IL-10 and glucocorticoids (GC) are usually regarded as critical anti-inflammatory agents (6-9).. Interleukin-10 (IL-10), the canonical anti-inflammatory cytokine, has also been pointed out as a central mediator in the late phase of sepsis

Around 50% of sepsis cases are associated to Gram-negative infections, where lipopolysaccharides (LPS; also known as endotoxins), a constituent of the bacterial outer membrane, have a central role throughout the development of the disease (10). While LPS initially induces an exacerbated production of pro-inflammatory cytokines leading to multiorgan failure and death resembling a septic shock, it is also capable of inducing endotoxin tolerance, an anti-inflammatory state that renders the host temporarily refractory to a subsequent lethal dose of LPS, which is the initial phase of the

immunosuppression found in septic patients (11). For these reasons, stimulation with LPS to induce inflammation and tolerance/immunosuppression constitute an attractive model to emulate the different phases of this disease.

There is a vast amount of literature on the role of IL-10 as an anti-inflammatory agent that mediates the restoration of homeostasis, but the relevance of this cytokine in the establishment and maintenance of immunosuppression is less clear. Elevated plasma levels of IL-10 in septic patients have been associated to the inhibition of pro-inflammatory cytokines and to the decrease in the expression of Human Leukocyte Antigen D Related (HLA-DR) in monocytes, a crucial marker of immunosuppression and poor prognosis (12). On the other hand, IL-10 was proven to be critical for survival in the early phases of sepsis (7, 13, 14, 15), but its role in the late phase is less clear. Muenzer *et al.* demonstrated that blocking IL-10 synthesis in the immunosuppression-phase of a cecal ligation and puncture (CLP) model improved the immune response and survival (16), although no correlation between the elevated IL-10 levels and survival has also been reported (17).

Regarding GC, we have previously demonstrated that the maintenance of both endotoxin tolerance and immunosuppression induced by LPS was dependent on the presence of GC, since both states can be reversed by mifepristone (RU486), an antagonist of GC receptors, by metyrapone, an inhibitor of GC synthesis, or by dehydroepiandrosterone (DHEA), a hormone with anti-GC properties (8, 9, 18). It is known that synthesis of IL-10 and GC are interrelated, and that one can modulate the induction of the other although this relationship is not always linear (19).

Taking into account that the role of these anti-inflammatory agents have been usually studied individually, the aim of this study was to evaluate the role of IL-10 in the pro- and anti-inflammatory events induced by LPS, with particular emphasis in the relationship of this cytokine with GC. For this purpose, using a mouse model of IL-10 knockout mice (IL-10 KO), we attempted to understand the relevance, redundancy, or hierarchy of these pivotal anti-inflammatory agents in the restoration of homeostasis in animals challenged with endotoxins.

The current work has been structured as follows: 1) evaluation of the role of IL-10 upon LPS challenge (endotoxic shock or inflammatory phase); 2) relevance of GC in the absence of IL-10; 3) evaluation of the roles of IL-10 and GC in the establishment and maintenance of endotoxin tolerance (immunosuppression/tolerance phase).

MATERIALS AND METHODS

Animals

Wild-type (WT) BALB/c and IL-10 knockout (IL-10 KO) (C.129P2 (B6)-IL10 ^{tm1Cgn}/J) BALB/c male mice (8-12 weeks old), were provided by Leloir Institute Foundation, Buenos Aires, Argentina. Animals were maintained under a 12-h light–dark cycle at 22±2 °C and fed with standard diet and water *ad libitum*. All the experiments were performed between 8 and 10 a.m. Animals were bred and housed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Institute of Experimental Medicine (IMEX)-CONICET-ANM. All animal procedures followed the Guide for the Care and Use of Laboratory Animals (20).

Reagents

LPS from *Escherichia coli* O111:B4 was obtained from Sigma, St Louis, MO, USA. Dexamethasone was from Sidus, Buenos Aires, Argentina. Antibodies: fluorescein isothiocyanate (FITC)-Anti-TNF-RI from Abcam, Cambridge, MA, USA; Phycoerythrin (PE)-Anti-TNF-RII from Bio Legend, USA. Mifepristone [RU486-17-hydroxy- 11-(4-dimethylaminophenyl) 17-(1-propynyl) estra-4, 9-diene-3-one] was obtained from Sigma-Aldrich (St Louis, MO, USA). RU486 was dissolved in propyleneglycol (PEG, vehicle). Corticosterone [^{3}H] (Cort-[^{3}H]) in ethanol was provided from New England Nuclear (Boston, MA, USA) with a specific activity of 78.1Ci / mM (2.89 TBq / mM).

Lethality studies

Mice were challenged intraperitoneally (*i.p.*) with different doses of LPS. Decreasing doses of LPS, starting from lethal dose for a WT mice (2 LD50 = 200 μg/mouse) followed by 100, 50, 10, 5, 1 and 0.25 μg/mouse, were inoculated to each group. Mortality was evaluated up to 72 h after each dose, considering euthanasia by CO_2 inhalation as the experimental end-point determined by clinical score evaluation.

Mouse Models

Endotoxic shock: mice were challenged with LPS 100 μg/mouse, *i.p*. and at different times after challenge (0, 1.5, 3 and 6 h) whole blood and plasma were collected. Control groups of animals were inoculated with phosphate-buffered saline (PBS) (naive group). Livers and kidneys were removed 6h after LPS inoculation (described below in *Histology and immunohistochemistry* Section).

Dexamethasone pretreatment: mice were pretreated with the synthetic glucocorticoid dexamethasone (DEX, 62.5µg/mouse, *i.p.*) or PBS, 20 minutes before the inoculation with LPS (µg/mouse, *i.p.*). (5, 50 or 200). At 90 minutes after the LPS, plasma was collected and mortality was evaluated.

Endotoxin tolerance: mice were inoculated daily with different doses of LPS (*i.p.*) for a period of 10 days. Inoculation regimen was (µg/mouse): 0.25 for the first 2 days, 0.5 for the following 2 days, 1.0 for 2 days, 5.0 for 2 days and 10 for the last 2 days (schematically represented in Fig. 4b and Fig 5a). Throughout this scheme, at different times the plasma samples were collected for cytokines and corticosterone determination. The adrenal glands from tolerized mice were removed 24h after the last LPS dose.

Treatment with Mifepristone (RU486): a) Tolerance Maintenance. Tolerized WT and IL-10 KO mice, were inoculated with RU486 (300µg/dose/mouse; subcutaneously (s.c.) 24 h after of the last dose of LPS. Thirty minutes later, each group was challenged with their lethal dose of LPS (200ug/mouse for WT and 10ug/mouse for IL-10 KO mice). A control group of tolerant IL-10 KO mice was treated with PEG (RU486 vehicle) and 30 minutes later, were challenged with the lethal dose of LPS. *b) Tolerance Establishment.* WT and IL-10 KO mice were tolerized with the scheme described above, but 30 minutes before each LPS dose they were treated with RU486 (300µg/dose/mouse; s.c.). Twenty four hours after the last tolerizing dose of LPS, the animals were challenged with the lethal dose of LPS corresponding to each strain. The control group of IL-10 KO mice was treated with PEG. Mortality was evaluated up to 24 h

The number of animals per group was 4 or 5 depending on the experiment (see Legend to each Figure). At least two experimental repetitions were performed to reach a total number of 8 or 10 animals per group.

Determination of TNF-α receptors in blood leukocytes

Whole blood (100 µl) was incubated for 30 minutes at 4°C with (FITC)-anti-TNF-RI and (PE)-anti-TNF-RII antibodies. Cells were treated with erythrocyte lysis buffer and the expression of TNF-α receptors in different leukocytes populations was evaluated in a Becton Dickinson FACScan flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Biochemical parameters

Alanine aminotransferase (ALT), aspartate transaminase (AST) and creatinine (Cre) levels were determined in plasma with a commercially available kit from BioTécnica (Varginha, Minas Gerais, Brazil) in a MINDRAY BS-200E clinical chemistry auto analyzer according to the manufacturer's instructions.

Leukocyte Counts

Blood cells obtained by submandibular puncture in heparinized tubes were differentially counted microscopically (mononuclear and polymorphonuclear cells) in a Neubauer chamber with Turk's solution (Merck).

Radioimmunoassay (RIA)

Corticosterone levels were determined in plasma by a commercially available radioimmunoassay (RIA) kit from ICN Biomedicals (Costa Mesa, CA, USA), according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

Levels in plasma of TNF-α, IFN-γ, IL-12, IL-6, TGF-β and IL-10 were performed by ELISA assay (OptEIA set; BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Histology and immunohistochemistry

For collection of liver, renal and adrenal gland specimens animals were euthanized by CO_2 inhalation. The organs were carefully dissected and whole adrenal glands, liver and decapsulated kidney portions were fixed in PBS with 10% formaldehyde (pH 7.2); followed by paraffin embedding according to our standard protocol (21). Longitudinal sections (3 μm thick) of paraffin embedded organs were stained by hematoxylin-eosin and periodic acid Schiff (PAS) methods and used for histological evaluation of hepatic and renal damage and morphometrical assessment of adrenal cortex structural changes induced by LPS treatment.

For evaluation of tissue pro-inflammatory status, kidney and liver samples were immunolabeled using a goat polyclonal antibody against rat tumor necrosis factor-alpha (TNF-α) (Cat. #af-510-na, R&D Systems, Minneapolis, MN, USA) at a 1:400 dilution in PBS. Immunolabeling of specimens was carried out using a modified avidin-biotin-peroxidase technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, CA, USA) as described previously (22). For histomorphometric evaluation all observations were made with a light microscope Nikon E400 (Nikon Instrument Group, Melville, New York. USA). Tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the animal belonged. All measurements were carried out using an image analyzer Image-Pro Plus ver. 4.5 for windows (Media Cybernetics, LP. Silver Spring, MD. USA). The samples kidney and liver were assessed in each animal (n= 6) on 10 consecutive microscopic fields at 400x magnification, where each field represents 1.13 mm^2, resulting in a total

explored area of 11.3 mm^2. The immunolocalization of tissue TNF-α and staining PAS+ was expressed as percentage of area with positive staining TNF-α or PAS / mm^2.

Morphometrical analyses

Photo-documentation was performed using the Nikon E400 (Nikon Instrument Group, Melville, NY, USA) microscope system. All measurements were made on one set of 5 serial parallel sections taken through the center of the adrenal gland and perpendicular to its flat (renal) side. Quantitative analyses were performed on a set of at least four random images taken at x200 magnification taken from each animal using Image J software (National Institutes of Health, https://imagej.nih.gov/). Briefly, measurements were performed by drawing a line parallel to the base of each image, identifying the midpoint of this line, and then marking a perpendicular line encompassing the zone fasciculata (zf, identified morphologically), and then extending it to the medullar and capsular edges to encompass the whole with of the adrenal cortex (C). The proportional width occupied by the zone fasciculata was calculated as the ratio of these lines (i.e., zf : C) and expressed as mean percentage. For immunohistochemical analyses, staining localization was assessed by two independent observers and photo-documented at a x400 magnification.

Statistical analysis

Values are expressed as the mean ± SEM of n observations. The assumption test to determine the Gaussian distribution was performed by the Kolmogorov and Smirnov method. For parameters with a Gaussian distribution, the differences between 2 groups were assessed by unpaired Student's t test. Differences between more than 2 groups were assessed by a one-way ANOVA followed by a Tukey's multiple comparison tests. Production kinetics differences between more than 2 groups were assessed

by a two-way ANOVA followed by a Bonferroni's multiple comparison test. The differences in the width ratio of zone fasciculata / cortex among more than 2 groups were assessed by a two-way ANOVA followed by Bonferroni post-hoc comparisons. For parameters with a non-Gaussian distribution, such as morphological quantification analyses, comparisons between 2 groups were performed using Mann-Whitney test. All statistical tests were interpreted in a two-tailed fashion and a P-value<0.05 was considered statistically significant.

RESULTS

IL-10 KO mice showed an enhanced sensitivity to LPS

To determine the sensitivity to endotoxin in the presence or absence of IL-10 in our experimental conditions, we evaluated different LPS doses *in vivo*. For this purpose, wild type (WT) and IL-10 deficient (IL-10 KO) BALB/c mice were *i.p.* injected with different amounts of LPS and the survival was followed up to 72 h after challenge. The results shown in Table 1 indicate that IL-10 KO mice were, approximately, 200 times more sensitive to LPS effects than WT mice.

IL-10 KO mice showed exacerbated secretion of cytokines after LPS challenge

Considering that IL-10 is one of the pivotal anti-inflammatory agents that inhibit the production of pro-inflammatory cytokines in infectious conditions (23, 24), we evaluated the production and kinetics of different critical cytokines following endotoxin challenge. Then, WT and IL-10 KO mice were *i.p.* injected with 100µg/mouse of LPS and relevant cytokines associated to a septic process such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), transforming growth factor-beta (TGF-β) and interleukins (IL-12, IL-6, and IL-10) were evaluated in plasma at 0, 1.5, 3 and 6 h after LPS inoculation.

As depicted in Fig. 1a IL-10 KO mice had significantly higher levels of TNF-α than WT mice at each time after LPS challenge. The increase of TNF-α, a central pro-inflammatory mediator in sepsis (6, 23), was sustained in IL-10 KO mice for at least 6 h after endotoxin stimulus, whereas WT mice exhibited a transient increase at 1.5 h post LPS challenge, followed by a decrease that was maintained up to the end of the experiment.

In addition, the plasma concentration of pro-inflammatory cytokines IL-12 and IFN-γ were increased at 3 and 6 h respectively after LPS injection, being significantly greater in IL-10 KO mice (Fig. 1b, c). IL-6 levels, a cytokine with pro- and anti-inflammatory functions, were similar between WT and IL-10 KO mice (Fig. 1d). On the other hand, significantly high levels of the anti-inflammatory TGF-β were found in plasma of IL-10 KO mice 6 h after endotoxin injection (Fig. 1e). As expected, IL-10 was not detectable in IL-10 KO mice (Fig. 1f).

IL-10 KO mice showed an increased percentage of leukocytes expressing TNF-α receptors after LPS challenge

Considering that elevated levels of TNF-α were associated with an increased sensitivity to endotoxin in IL-10 KO mice, and taking into account that the activity of TNF-α is dependent on the levels of its specific receptors, we evaluated the expression of the two TNF-α receptors (TNF-R1 -p55- and TNF-R2 -p75-) on the membrane of peripheral blood leukocytes from WT and IL-10 KO mice, at 0 and 6 h after endotoxin challenge.

The results show that although TNF-RI and TNF-RII were mainly expressed in monocytes and polymorphonuclear cells (PMN) of WT and IL-10 KO, no significant differences were observed in the basal expression of these receptors (Supplementary Figure 1, http://links.lww.com/SHK/A798).

However, LPS challenge induced an increase in the percentage of PMN expressing TNF-RI only in IL-10 KO mice (Suppl. Fig.1a, http://links.lww.com/SHK/A798), while for TNF-RII an increased percentage in PMN of WT mice was found, also observing a significantly higher percentage in both monocytes and PMN in IL-10 KO mice (Suppl. Fig.1b, http://links.lww.com/SHK/A798). Similar results were obtained by the mean of fluorescence intensity (Suppl. Fig.1d, http://links.lww.com/SHK/A798).

IL-10 KO mice displayed enhanced multiple organ damage than WT mice

The exacerbated inflammatory response induced by LPS causes damage to multiple organs, including liver and kidney (25, 26). Therefore, we evaluated biochemical parameters associated with tissue damage at 3 and 6 h after LPS challenge. For this purpose, plasma levels of ALT and AST, markers of liver damage, and creatinine (Cre), an indicator of renal failure, were measured.

WT and IL-10 KO mice had similar basal level of enzymes and Cre (Fig. 2). However, following endotoxin challenge, IL-10 KO mice showed an earlier increase of AST, being elevated 3 h after LPS injection. In addition, after 6 h from LPS challenge, AST was significantly elevated in both WT and KO IL-10 mice, being considerably higher in the latter (Fig. 2a). On the other hand, plasma levels of ALT and Cre showed a comparable increase in WT and IL-10 KO mice in each point, being significantly high 6 h after LPS (Fig. 2b and c).

Taking into account that leukocytes play a role in the inflammatory process of an endotoxic shock, we evaluated the circulating number of leukocytes in treated mice. Peripheral blood cell count showed a significant decrease in mononuclear and PMN cells 6 h after LPS challenge both in WT and IL-10 KO mice, with a greater reduction in PMN of IL-10 KO mice, despite having comparable basal levels of

both cell types (Suppl. Table 1, http://links.lww.com/SHK/A799). This could be associated with recruitment of peripheral blood leukocytes into the tissues, which would lead to the above mentioned increase in damage markers.

In accordance with the biochemical findings, our histological analyses revealed more histopathological damage in IL-10 KO mice after LPS challenge. In liver sections, IL-10 KO mice exhibited a remarkable depletion of PAS^+ cells compared to WT animals, indicative of a decreased glycogen storage capacity of hepatic cells (Fig. 2d and 2f). In addition, a marked increase in the expression of the pro-inflammatory cytokine TNF-α was observed both in the liver and kidney of LPS-challenged IL-10 KO mice (Fig. 2e, 2g and 2h). In contrast, renal and liver histology in untreated WT and IL-10 KO mice were similar and displayed no signs of abnormalities (data not shown).

Endotoxin induced exacerbated levels of plasma corticosterone in IL-10 KO mice

Considering that endotoxins induce an increase of serum GC through activation of the hypothalamic–pituitary–adrenal axis, it was important to evaluate the corticosterone levels in WT and IL-10 KO mice after an LPS challenge. Thus, at different times after LPS injection (0, 1.5 and 3 h) plasma were collected and corticosterone was evaluated. Our results suggest that IL-10 KO mice have a slightly higher level of corticosterone in basal condition. However, although after LPS challenge the corticosterone levels were increased in both mouse strains, IL-10 KO mice showed significantly higher levels at different times after LPS injection (Fig. 3a).

Dexamethasone induced a partial refractoriness to LPS challenge in IL-10 KO mice

GC are strong inhibitors of proinflammatory mediators such as TNF-α, and are crucial agents in the refractoriness to LPS in tolerant WT mice (8, 9, 18). In order to investigate whether the absence of IL-10 exerts changes on the corticoid-mediated protection in the naive mice to a lethal dose of LPS, we evaluated the effect of a synthetic GC, Dexamethasone (DEX), in IL-10 KO compared to WT mice.

Thus, WT and IL-10 KO mice were pretreated with DEX and then challenged with different doses of LPS (200ug LPS: lethal for both WT and KO mice; 50ug LPS: sub-lethal for WT and lethal for KO mice; or 5ug LPS: non-lethal for WT and lethal for KO mice), and the mortality was evaluated. DEX pretreatment protected WT but not IL-10 KO mice, against a lethal injection of LPS. The protective effect of DEX in IL-10 KO mice could only be observed using very low doses of LPS (Table 2).

Considering that the decrease in plasma TNF-α level is an indicator of the anti-inflammatory effect of DEX after LPS challenge, we evaluated the levels of this cytokine in plasma 1.5 h after each dose of LPS.

The results showed that DEX induced a strong anti-inflammatory effect on WT mice, reflected in the low level of TNF-α detected (Fig. 3b) and in the refractoriness against the lethal dose (200µg LPS) (as described in Table 2). However, in IL-10 KO mice although DEX pretreatment decreased TNF-α levels induced by LPS, even lower than those observed in WT without DEX pretreatment +LPS 50µg, when IL-10 KO mice were inoculated with the same LPS dose did not survive the endotoxin challenge (Fig. 3c, Table 2). Only when the dose of LPS was further reduced (5µg LPS) the DEX-pretreated IL-10 KO mice showed refractoriness to the endotoxin (Fig. 3d, Table 2). This shows that DEX promotes a full anti-inflammatory effect in the absence of IL-10 only at very low doses of LPS.

Endotoxin tolerance phenomenon was established in absence of IL-10

Next, we studied the role of IL-10 in endotoxin tolerance, which is considered the state that precedes immunosuppression in sepsis (11) and is characterized by a decreased production of pro-inflammatory mediators along with an increased secretion of the anti-inflammatory cytokines, such as IL-10. Thus, tolerant WT mice showed an increase in the IL-10 plasma levels 8 h after endotoxin challenge (Fig. 4a). Taking this into account, our purpose was to analyze if it is possible to establish tolerance in the absence of IL-10. In addition, since decreasing TNF-α levels is a key marker of the establishment of tolerance phenomenon, we evaluated the production of this cytokine in plasma during the process.

Thus, while an exacerbated increase of TNF-α in IL-10 KO mice after the first tolerizing dose of LPS was detected, the kinetics throughout the days was similar to that obtained in WT tolerant mice (Fig. 4b). This suggests that even in the absence of IL-10 it is possible to establish the tolerance phenomenon, although the robustness was different. In fact, while tolerant IL-10 KO mice became resistant to a lethal dose of LPS for this mouse strain (10ug), when these animals were challenged with a lethal dose for WT mice (200ug LPS) the mortality was 100%, indicating that although a level of tolerance is reached in IL-10 KO, it is not established with the same strength than in WT mice (mortality of tolerant mice after challenge with 200 μg LPS/mouse: WT: 0/6 vs. IL-10 KO: 6/6).

Tolerant IL-10 KO mice showed exacerbated levels of plasma corticosterone

Taking into account it was possible to establish tolerance in IL-10 KO mice and having demonstrated the relevance of GC in the maintenance of both LPS-induced tolerance and immunosuppression (8, 9, 18), we consider it is important to evaluate the plasma corticosterone levels during tolerance establishment in IL10 KO and WT mice. Thus, at different days during the tolerizing scheme and at 3

h after each LPS injection, plasma were collected and corticosterone was evaluated (Fig. 5a). The results showed a sustained increase in corticosterone levels in both strains, being significantly increased in IL-10 KO mice.

Considering the significantly exacerbated levels of GC found in LPS tolerant IL-10 KO mice the adrenal gland histology was studied. Thus, we evaluated the width of the zone fasciculata (as % cortex width), the glucocorticoid-producing region, by a morphometrical analysis as indicated in Materials and Methods. The results in the Figures 5b and 5c showed a significantly increase of the relative width of the zone fasciculata in LPS tolerant IL-10 KO mice, suggesting an association between this histological change and the exacerbated corticosterone levels observed in plasma.

We have previously demonstrated that treatment with Mifepristone (RU486), a GC receptor antagonist, is capable to overcome the LPS-induced tolerance, suggesting a central role for GC in the maintenance of endotoxin tolerance (8, 18). Taking into account this, we assessed whether the elevated corticosterone levels in tolerant IL-10 KO mice could be contributing to partial tolerance, evaluating its role both in the maintenance and establishment of tolerance.

For this, the tolerant or naive mice of both strains were treated with different administrations of RU486 and challenged with their corresponding lethal dose of LPS as described in the *Materials and Methods* section. Mortality was evaluated up to 24 h post-LPS.

In the maintenance phase, as expected for WT mice, the tolerance disruption by RU486 occurred 24 h after LPS administration, whereas in IL10 KO disruption developed considerably earlier, being 6 h after endotoxin challenge (Table 3). In establishment phase, when IL10 KO mice were treated with RU486 and a tolerizing dose LPS simultaneously, mice showed signs associated with a greater

inflammation such as tremor, diarrhea, lethargy and bristled hair, throughout of the all the tolerance establishment, reaching a mortality rate of 40%. Contrary, WT mice tolerized simultaneously with RU486 were 100% tolerant, in accordance with our previous results (18) (Table 3).

DISCUSSION

Endotoxin-based animal models enable to mimic different phases that occur during a septic process. LPS is capable of both promoting an overwhelming inflammatory response which resembles the early septic shock, and also inducing an anti-inflammatory state known as endotoxin tolerance which, frequently concludes in a late severe immunosuppression, where most deaths occur. In the present work, we attempted to clarify the role of two mediators with widely accepted anti-inflammatory and immunosuppressive properties, namely IL-10 and GCs, evaluating them during both LPS induced phases.

In this report we observed a higher sensitivity to endotoxin (at least 200 times) in IL-10 KO compared to WT mice. This is in agreement with other authors that demonstrated an increased lethality to endotoxemia by IL-10 neutralization (27, 28).

This was paralleled by high levels of proinflammatory cytokines such as TNF-α, IFN-γ and IL-12 in IL-10 KO mice after LPS injection, which is in line with the role of IL-10 in limiting the secretion of these cytokines in the initial phase of sepsis. Moreover, the elevated and sustained levels of plasma TNF-α observed for at least 6 h in IL-10 KO mice after LPS injection, indicate that IL-10 is essential as an anti-inflammatory mediator to control/modulate both the levels and the kinetics of this cytokine.

In addition, the increase in the expression in monocytes and PMN of TNFRII in IL-10 KO mice after endotoxemia suggest that IL-10 could modulate not only the agonist (TNF-α), but also the TNF-Receptor levels on the cell surface. The functional relevance of these differential regulations in WT and IL-10 KO mice should be demonstrated with further studies.

The inflammatory processes induced by LPS can provoke renal and hepatic injury which could lead to shock and subsequently to a multiple organ dysfunction syndrome (MODS) (29). In this study we observed an increase of damage markers in both strains after LPS challenge, being only AST significantly higher in IL-10 KO mice. However, kidney and liver histology showed a greater inflammatory process and markers indicative of enhanced liver damage in IL-10 KO mice following LPS challenge, which could be associated, at least in part, with their increased lethality.

Concerning the relationship between IL-10 and GC, we found an increase of corticosterone in both IL-10 KO and WT mice after LPS stimulus, although this induction was significantly higher in KO compared to WT animals. This result can be interpreted as a compensatory effect due to the absence of IL-10, an effective anti-inflammatory mediator, to reduce early inflammatory events. In the same line, we observed an increase of TGF-β, another anti-inflammatory mediator involved in the inhibition of TNF-α in a similar manner to IL-10 (6). Furthermore, since high levels of corticosterone were also observed under basal conditions of IL-10 KO mice, all this together allows us to suggest that IL-10 exerts a negative regulation on GC levels, as has been mentioned by others (19).

However, we found that pretreatment with DEX only induced a strong refractoriness to a lethal dose of LPS in WT but not in IL-10 KO animals indicating that GC were not able to fully exert its anti-inflammatory/protective role against endotoxin challenge when IL-10 was missing.

This outcome could have at least two considerations. The role of TNF-α in the mortality during hyperinflammatory phenomena is well-recognized. Our results are consistent with a lethal effect of this cytokine, as IL-10 KO mice challenged with LPS secreted more TNF-α than WT mice. However, the levels did not correlate with mortality, because DEX was able to greatly inhibit TNF-α in IL-10 KO mice + 50μg LPS but this reduction did not translate in enhanced survival, suggesting that other mediators can be involved. Alternatively, IL-10 KO mice can be more sensitized to TNF-α, due to a greater number of TNF-α / TNF-Rs interactions which could overtake the refractoriness induced by DEX. The absence of IL-10 seems to be more critical than the reduction of TNF-α levels, but further studies are required to confirm this hypothesis and the exact role of TNF-α in the mortality of IL-10 KO mice.

As it is well-known, a partial resolution of the initial inflammatory response is necessary to induce the tolerance phenomenon. In this study, while tolerance was achieved in absence of IL-10, it was possible only by using very low doses of LPS during tolerization, avoiding the exacerbated initial inflammatory response, which promotes endotoxin lethality. In addition, the robustness of the tolerance proved to be extremely weak since it only supported low doses of LPS challenge. When tolerant IL-10 KO mice were challenged with the lethal dose of WT animals, tolerance rupture was achieved.

Tolerant IL-10 KO mice showed elevated levels of GC in plasma together with a relative enlargement of the adrenal zone fasciculata. Paradoxically, these levels were not enough to compensate the

exacerbated inflammatory response, since IL-10 KO mice showed an increased sensitivity to LPS challenge. However, when GC receptors were blocked by RU486, it not only drove the earlier disruption of tolerance maintenance, but it also partially prevented the tolerance establishment in IL-10 KO mice. These results suggest that in IL-10 absence, the endogenous GCs could lead a pivotal role in both phases of endotoxin tolerance phenomenon. It is interesting to note that despite we observed that in the tolerance establishment plus RU486 the mice showed signs of exacerbated inflammation, it did not translate into mortality in all animals, and further studies are needed to fully understand the relationship between IL-10 and GC in the establishment of tolerance.

These observations allow us to consider that during an early inflammatory process, IL-10 would be acting as a critical regulator for the resolution of acute inflammation, for example, by modulating both the levels and the biological activity of TNF-α as shown in our results. Thus, we could speculate that elevated GC levels during the initial phase of an inflammatory event would be associated with the induction of anti-inflammatory mediators such as IL-10, TGF-β, rather than to direct modulation of the inflammatory cascade. Furthermore, although IL-10 does not appear to be essential to establish the endotoxin tolerance, its absence leads to a weak tolerance in which the GC were unable to proceed as a compensatory mediator for inhibiting the development of an acute inflammation, although they would seem to play a pivotal role in the development of partial tolerance.

An important limitation of our work is that in order to conclusively attribute the observed differences in IL-10 KO mice to the absence of this cytokine, and not to a compensatory effect, an alternative blockade method should have been tested. However, extensive literature supports the IL-10 KO model in general, and even its involvement in LPS-induced inflammation in particular (27, 28, 30-32).

Finally, our results demonstrate the importance of the complementary action between IL-10 and GC, which may help to understand in part, the complexity of shock and/or tolerance/immunosuppression induced in sepsis, and can eventually contribute to implementing future therapies on a more rational basis.

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AUTHOR CONTRIBUTION

B. Rearte conceived and designed the experiments. M. Cordoba Moreno, M. F. Todero, A. Fontanals, G. Pineda, M.V. Ramos, and B. Rearte performed the experiments. M. Cordoba Moreno, M. F. Todero, M.A. Isturiz and B. Rearte analyzed and discussed the data. Adriana Fontanals and D. Montagna contributed with the management and procedures performed with mice. G. Barrientos and J.E. Toblli designed, performed and analyzed the histology and immunohistochemistry experiments. G. Pineda, D. Montagna, M.V. Ramos and M.F. Todero contributed with reagents/materials/analysis tools. N. Yokobori contributed with the writing and the discussion of the results. B. Rearte and M. A. Isturiz wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Cytokine production kinetics in plasma after LPS challenge. WT and IL-10 KO mice were inoculated with LPS (100µg) and at different times after challenge (0, 1.5, 3 and 6 hours) the plasma was collected and the cytokines levels were evaluated through an ELISA assay. (a) TNF-α, (b) IFN-γ, (c) IL-12, (d) IL-6, (e) TGF-ß and (f) IL-10. Each point represents the mean ± SEM of n=5 mice per group. The experiment is representative of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with WT mice at the same time. WT-LPS: WT mice LPS-treated; IL-10KO-LPS: IL-10 KO mice LPS-treated.

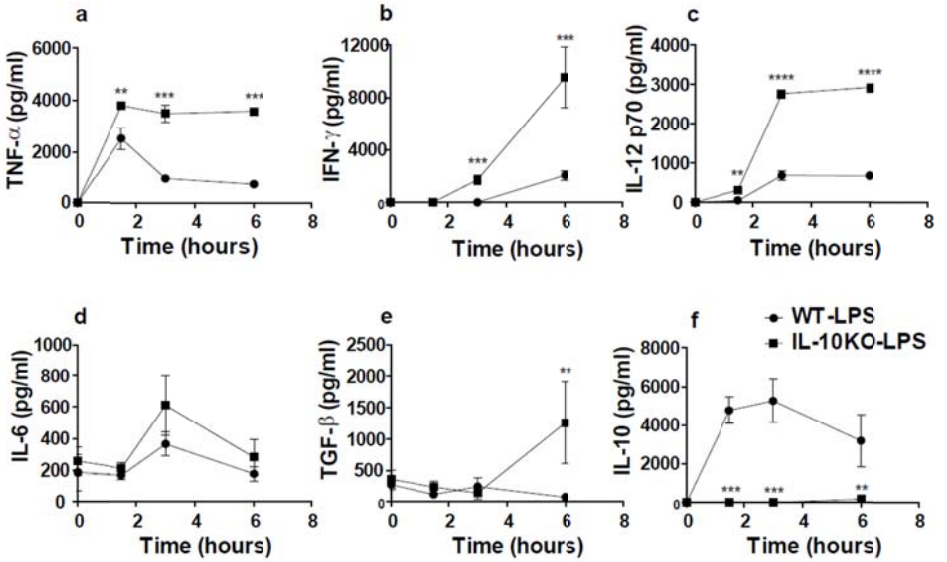

Figure 2. Plasma biochemical parameters and histopathology after LPS challenge. WT and IL-10 KO mice were inoculated with LPS (100 µg) and at different times after challenge (0, 3 and 6 hours) the plasma was collected and enzymatic levels were evaluated. In the last time-point, the liver and kidney were removed for histopathological analysis. (a) Aspartate transaminase (AST), (b) alanine transaminase (ALT) and (c) creatinine (Cre). Each point represents the mean ±SEM of n=5 mice per group. The experiment is representative of at least two independent experiments. ɔP<0.05 compared with WT-LPS mice at the same time. *P<0.05; ***P<0.001; ****P<0.0001 compared with the same mice at time zero. & P<0.001 compared with the same mice at 3 hours. (d) Liver sections stained by PAS showed a marked depletion of glycogen storage after endotoxemia in IL-10 KO mice compared form WT mice (arrows: PAS+ hepatocytes with glycogen storage). (e) Immunohistochemistry assay both in liver and kidney showed markedly increased expression of the pro-inflammatory cytokine TNF-α in IL-10 KO mice compared with WT mice after endotoxin (arrows: positive labeling for TNF-α). (Original magnification 400X). (f, g h) Histological score: Evaluation was made by light microscopy carried out independently by two investigators blinded to sample identity in each animal (n= 6) as indicated in *Materials and Methods*. *P<0.01 compared with WT-LPS mice. WT-LPS: WT mice LPS-treated; IL-10KO-LPS: IL10KO mice LPS-treated.

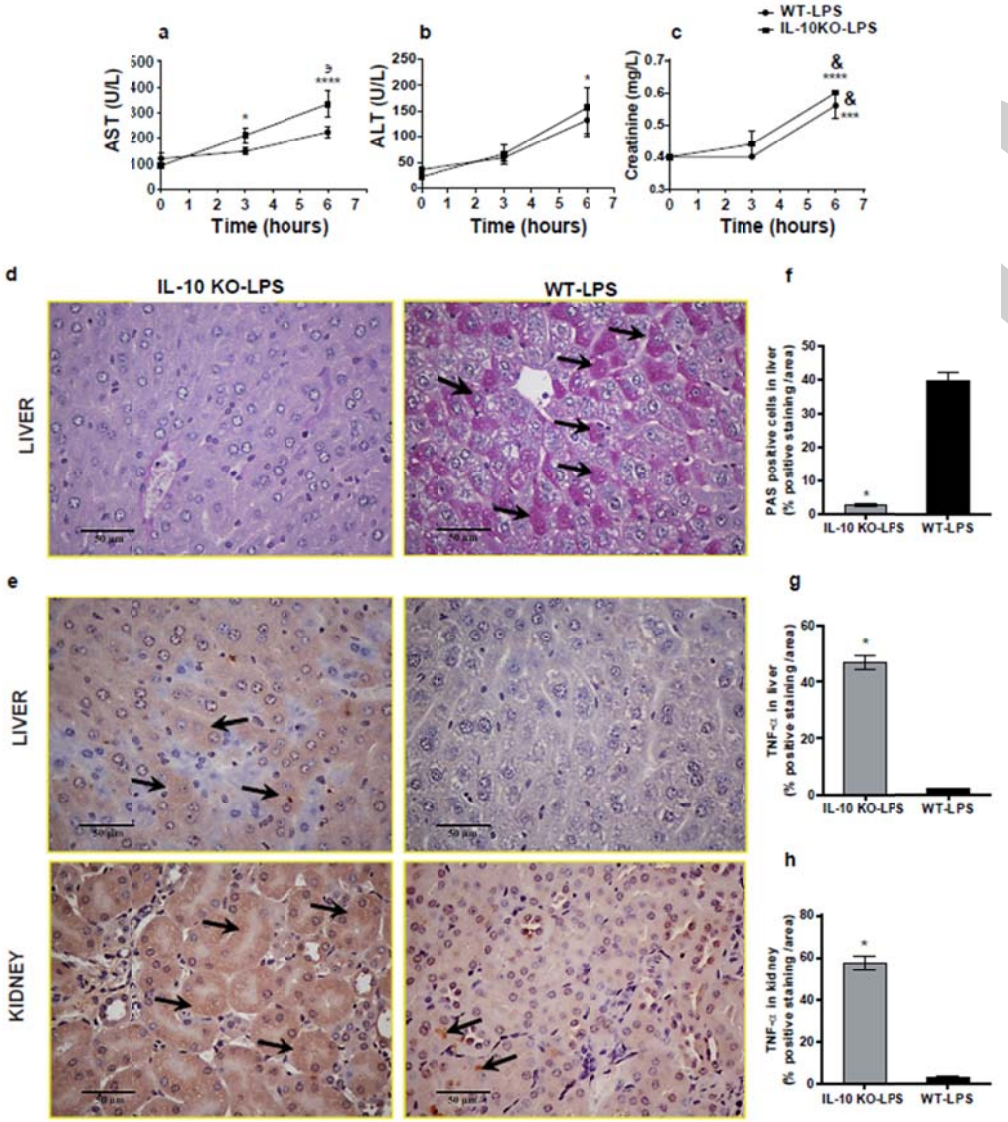

Figure 3. Endogenous corticosterone levels and the effect of exogenous glucocorticoids in WT and IL-10 KO mice challenged with LPS. (a) WT and IL-10 KO mice were inoculated with LPS (100μg) and at different times after challenge (0, 1.5 and 3 hours) the plasma was collected. Corticosterone levels were evaluated through a Radioimmunoassay (RIA). Each point represents the mean ± SEM of n=5 mice per group. Experiment is representative of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with WT mice at the same time. ɔP<0.01 compared between basal condition. WT-LPS: WT mice treated with one dose of LPS; IL-10KO-LPS: IL-10 KO mice treated with one LPS dose. (---) basal levels in PBS-treated WT mice. WT and IL-10 KO mice were pretreated with dexamethasone (DEX, 62.5 μg/mouse) or PBS, and 20 minutes after, were challenged with (b) 200, (c) 50 and (d) 5 μg/mouse of LPS. Plasma were collected at 1.5 h after the endotoxin inoculation. The level of TNF-α was evaluated through an ELISA test. Results are expressed as the mean ± SEM of n=4 mice per group. The experiment is representative of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001, ɔP<0.05. DEX+LPS: mice pretreated with DEX and challenged with LPS; LPS: mice pretreated with PBS and challenged with LPS.

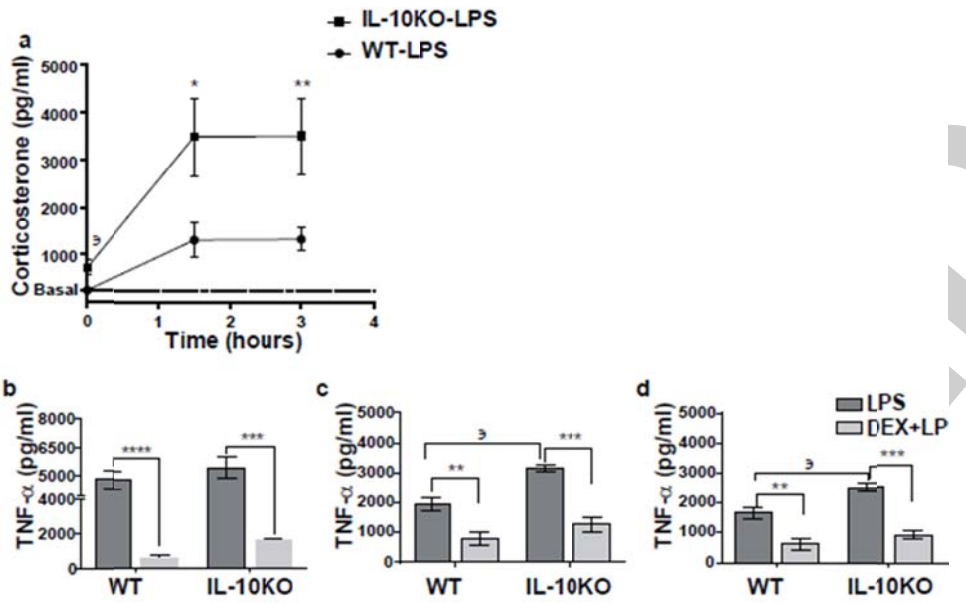

Figure 4. Plasma IL-10 and TNF-α levels in LPS tolerant mice. WT and IL-10KO mice were tolerized with different doses of LPS as indicated in Materials and Methods. (a) IL-10 levels in tolerant WT mice (WT-TOL). Plasma samples were collected 1.5, 8 and 24 hours after the last dose of LPS (10ug). (b) TNF-α levels during establishment of tolerance in WT (WT-TOL) and IL-10KO mice (IL-10KO-TOL). Plasma samples were collected 1.5 hours after each dose of LPS at 1, 3, 5, 7 and 11 days with the indicated doses (black arrows). All the cytokines were evaluated through an ELISA test. Results are expressed as the mean ± SEM of n=4 mice per group. The experiment is representative of at least two independent experiments. *P<0.05; ****P<0.0001 compared with WT mice at the same time. WT-TOL: WT mice tolerized with LPS; IL-10 KO-TOL: IL-10 KO mice tolerized with LPS.

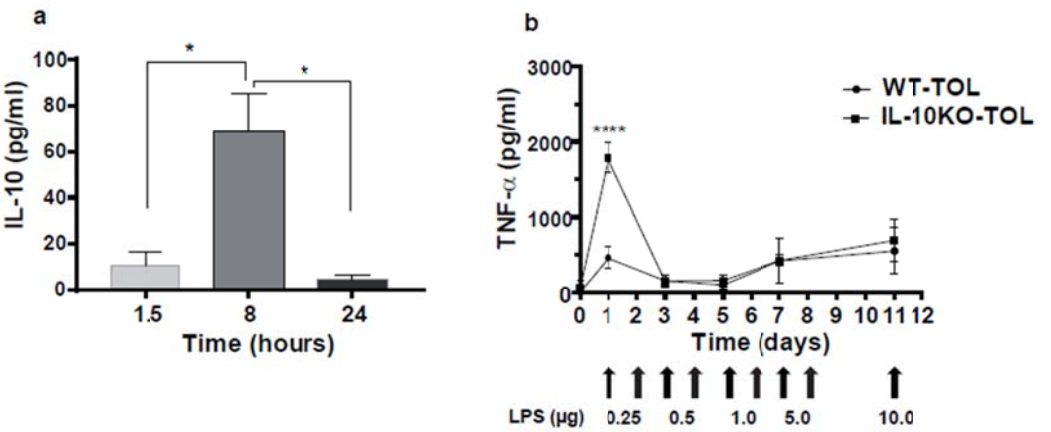

Figure 5. Corticosterone levels and histological changes in the adrenal gland during the establishment of tolerance in WT and IL-10 KO mice. (a) WT and IL-10 KO mice were tolerized with different doses of LPS (as indicated in Mat. and Met.) and at 3 hours after each dose of LPS in the following days (1, 3, 5, 7 and 11 days) (black arrows) the plasma was collected. Corticosterone levels were evaluated through a Radioimmunoassay (RIA). Each point represents the mean ± SEM of n=5 mice per group. The experiment is representative of at least two independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with WT mice at the same time. э P<0.01 compared between basal condition. (---) basal levels in PBS-treated WT mice. (b) Representative histological pictures of adrenal glands stained by hematoxylin-eosin. Abbreviations: zone glomerulosa (zg); zone fasciculata (zf); zone reticularis (zr); medulla (M). Magnification: x200 (c) Morphometrical analysis of the zone fasciculata width relative to the total cortex width. Results are expressed as the mean ± SEM. Each point represents the % zf width value of a random image obtained from at least 4 of each adrenal gland (n = 4 mice per group) as stated in Materials and Methods. *P<0.05 compared with LPS-Tolerant WT mice, **P<0.005 compared with PBS-treated IL-10 KO mice. WT-TOL: WT mice tolerized with LPS; IL-10KO-TOL: IL-10KO mice tolerized with LPS; WT: PBS-treated WT mice; IL-10 KO: PBS-treated IL-10 KO mice.

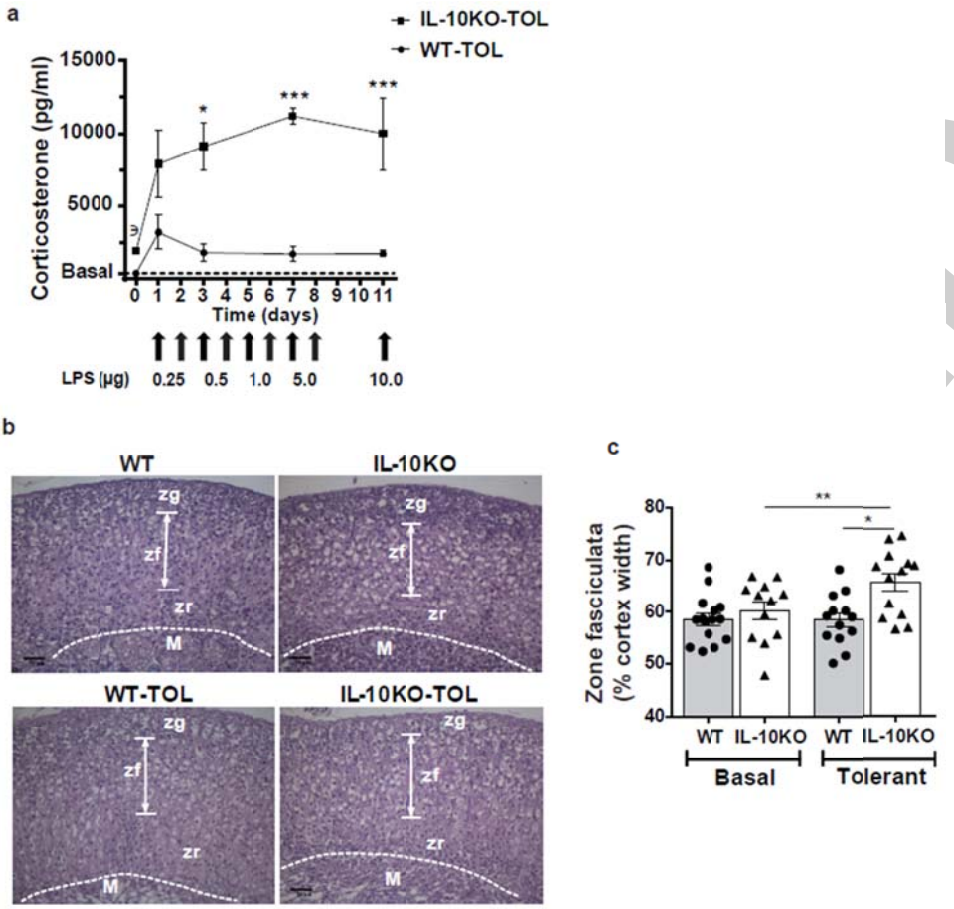

Table 1 Sensitivity of WT and IL-10 KO mice to different doses of LPS[a].

[LPS] µg/mouse	Mortality (%)	
	WT	IL-10 KO
200	100	100
100	45	100
50	0	100
10	0	100
5,0	0	100
1,0	0	100
0,25	0	0

[a]Data are representative of two separate experiments with similar results with n=5 mice per group.

Table 2 Refractoriness to dexamethasone pretreatment and LPS challenge in WT and IL-10KO mice[a]

[LPS] µg/mouse	% WT mortality		% IL-10KO mortality	
	LPS (n=4)	DEX +LPS (n=4)	LPS (n=4)	DEX + LPS (n=4)
200	100	0	100	100
50	0	0	100	100
5	0	0	75	0

[a]WT and IL-10KO mice were inoculated with LPS (*i.p.*) 20 minutes after to the dexamethasone pretreatment (DEX, 62.5 µg/mouse, *i.p.*) (DEX+LPS) or PBS (LPS) and the mortality was evaluated up to 48 hours later.

Table 3 RU486 effect in the maintenance and establishment of tolerance in WT and IL-10KO mice[a]

	Maintenance[a]		Establishment[b]	
	Tolerant + RU486		LPS_T + RU486	
Groups	WT(n=5)	IL-10 KO (n=5)	WT (n=5)	IL-10 KO (n=5)
Mortality %	100	100	0	40

[a] WT and IL-10 KO mice were tolerized as indicated in Materials and Methods. After 24 h of the last LPS dose, mice were inoculated with RU486 (300µg/mouse s.c.) and then with a lethal dose of LPS (200µg or 10µg /mouse, i.p.) respectively. [b] WT and IL-10 KO mice were treated simultaneously with a tolerizing LPS dose (LPS_T) and RU486 as indicated in Materials and Methods. After 24 h, mice were inoculated with each lethal dose of LPS respectively. The mortality was evaluated until 24 h after lethal LPS inoculation. Control groups:1) tolerant IL-10 KO mice were inoculated with propyleneglycol (PEG) (RU486 vehicle). 2) IL-10 KO mice treated with PEG and LPS_T . All animals survived a lethal dose of LPS (not shown)