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# Increased splenocyte proliferative response and cytokine production in β-endorphin-deficient mice

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**Abstract**

We used β-endorphin-deficient mice as a novel approach to confirm the physiological role that opioid peptides play in the development or regulation of the immune system. We found that mice lacking β-endorphin possessed an enhanced immune response, measured in terms of splenocyte proliferation and interleukin (IL)-2 mRNA levels, in vitro production of the splenic macrophage inflammatory cytokines IL-6 and Tumor Necrosis Factor (TNF)-α and plasma IL-6 following lipopolysaccharide (LPS) administration. β-Endorphin-deficient mice had attenuated increases of plasma ACTH and corticosterone levels in response to LPS. These results are consistent with a postulated inhibitory role of endogenous β-endorphin on the immune system at multiple levels.
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## 1. Introduction

The cross talk between immune and neuroendocrine systems is based on a complex interconnective network. This neuroendocrine–immune communication has been studied in recent years to acquire a more comprehensive view about several physiological and pathophysiological aspects of immunology in health and disease (Besedovsky and Del Rey, 1996; Blalock, 1989). In response to inflammation, there is an increase of pro-inflammatory cytokines, interleukin-1 (IL-1), IL-6, and Tumor Necrosis Factor (TNF) which act at different levels of the hypothalamic–pituitary–adrenal (HPA) axis to stimulate the synthesis and release of corticotrophin releasing factor (CRH), proopiomelanocortin (POMC) peptides such as β-endorphin and ACTH which are co-secreted by pituitary cells (Guillemin et al., 1977) and glucocorticoids (Bateman et al., 1989; Besedovsky and Del Rey, 1996; Marx et al., 1998) which exert their inhibitory actions on the immune system (Barnes, 1998; Bateman et al., 1989; Sapolsky et al., 2000; Wiegers et al., 1995, 2000), acting as a feedback control mechanism of the host inflammatory response. Other HPA neuropeptides and hormones such as β-endorphin also exert important immunological regulatory actions independent of glucocorticoids (Blalock, 1989; Jonhson et al., 1988; Panerai and Sacerdote, 1997; Roda et al., 1996).

POMC-derived peptides are produced not only by hypothalamic arcuate nucleus and anterior-intermediate pituitary cells, but also by different immunocompetent cells. POMC expression, translation, and processing have been demonstrated in mononuclear cells, spleen macrophages and lymph node cells (Buzzetti et al., 1989; Cabot et al., 1997; Lolait et al., 1986; Lyons and Blalock, 1997; Przewlocki et al., 1992). In particular, β-endorphin is synthesized and released in splenocytes, peripheral blood lymphocytes and monocytes (Buzzetti et al., 1989; Cabot et al., 1997; Lolait et al., 1986; Manfredi et al., 1995; Przewlocki et al., 1992; Smith and Blalock, 1981), a process enhanced by

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mitogens and anti-CD3-mediated activation (Kavelaars et al., 1991), β-adrenergic agonists (Kavelaars et al., 1990a), arginine-vasopressin and CRH (Kavelaars et al., 1989; Smith et al., 1986). Interestingly, CRH up-regulates the production of β-endorphin through an indirect mechanism that involves IL-1 secretion (Kavelaars et al., 1989). Moreover, inescapable intermittent footshock significantly increases β-endorphin concentration in splenocytes, peripheral blood mononuclear cells and lymph node cells in a CRH-dependent process (Sacerdote et al., 1994). Parallel to the HPA axis, glucocorticoids are able to down-regulate β-endorphin secretion from immune cells by blocking CRH-mediated production of β-endorphin (Kavelaars et al., 1990b).

There are substantial data concerning the effects of β-endorphin and opioids on the immune system, but a general agreement about the net effect has not been reached (Blalock, 1998; Morch and Pedersen, 1995; Panerai and Sacerdote, 1997; Roda et al., 1996; van den Bergh et al., 1991a). The important regulatory effects of β-endorphin on the immune system have been studied at different levels: mitogen-induced lymphocyte proliferation, chemotaxis, natural killer activity, and direct effects on cytokine and antibody secretion (Carr et al., 1990; Johnson et al., 1982; Mathews et al., 1983; Morch and Pedersen, 1995; Roda et al., 1996; Van Epps and Saland, 1984).

μ, κ, δ and orphan opioid receptors have been described in immunocompetent cells (Carr et al., 1989; Chuang et al., 1994, 1995; Halford et al., 1995; Radulescu et al., 1991; Sharp et al., 2000). Although μ-receptors are present in immunocytes, strong evidence exists for a naloxone-independent action of both β-endorphin and morphine on mitogen-induced proliferative responses of peripheral blood mononuclear cells (McCain et al., 1982; Puppo et al., 1985). δ-Opioid receptors are present on T cells, B cells, macrophages and NK cells. In the culture of macrophages, δ-opioid agonists induce an increase of cytokine release in response to bacterial lipopolysaccharide (LPS) (Gomez-Flores et al., 2001; House et al., 1996) and on $CD4^+$ and $CD8^+$ T cells, anti-CD3-induced proliferation is inhibited by δ-opioid receptor engagement (Shahabi and Sharp, 1995). Recent reports indicate that κ-selective agonists are able to inhibit the LPS-induced pro-inflammatory cytokine release by peritoneal macrophages and macrophage cell lines (Alicea et al., 1996; Belkowsky et al., 1995).

β-Endorphin potentiates LPS-induced IL-1 production by murine bone marrow-derived macrophages (Apte et al., 1990) and a Langerhans cell line XS52 (Hosoi et al., 1999) while morphine and δ agonists also augment LPS-induced TNF-α expression and release (Gomez-Flores et al., 2001; Peng et al., 2000). β-Endorphin stimulates basal levels of the IL-1 receptor antagonist (IL-1 ra), an effect apparently mediated by opioid receptors because it is blocked by naloxone treatment (Kovalovsky et al., 1999). Nevertheless, opposite effects with the same drugs have also been reported in primary cultures, macrophages cell lines and peripheral blood mononuclear cells (Alicea et al., 1996; Belkowsky et al., 1995; Chao et al., 1993).

Concerning T-lymphocyte activation, both stimulatory (Fontana et al., 1987; Gilman et al., 1982; Gilmore and Weiner, 1989; Heijnen et al., 1987; Kusnecov et al., 1987; van den Bergh et al., 1991b) and inhibitory (Manfredi et al., 1993; McCain et al., 1982; Panerai et al., 1995; Puppo et al.,1985) effects of β-endorphin on lymphocyte proliferation have been described. The action on Th1/Th2 cytokines is also controversial: both enhancement (Bessler et al., 1990; van den Bergh et al., 1991b) or inhibition (Garcia et al., 1992) of IL-2 production by β-endorphin were described. Also, stimulation of the Th1 cytokine IFN-γ was reported (Brown and Van Epps, 1986). The opioid antagonist naloxone has been shown to increase Th1 cytokine production in mice, suggesting that endogenous opioid peptides suppress Th1 cytokines (Panerai and Sacerdote, 1997; Sacerdote et al., 2000).

Although on the basis of the available data, β-endorphin seems to modulate immune functions in a predominantly negative way, divergent or even opposite effects have been noted even in the case of rigorously controlled models. Gene targeting to create null mutations is a powerful tool in modern biology and used to dissect complex phenotypes and clarify the function of individual gene products. In the present report, we analyzed several immunological parameters in mice lacking β-endorphin (Rubinstein et al., 1996) in order to study the role of β-endorphin in these regulatory pathways.

## 2. Materials and methods

### 2.1. Animal housing and genotyping

β-Endorphin wild type (WT, +/+) and β-endorphin-deficient (−/−) homozygous C57BL/6 mice of 8–10 weeks of age (25–30 g) were studied (Rubinstein et al., 1996). Animals were housed under standard lighting (lights on from 6:00 AM–6:00 PM) and temperature (22 ± 2 °C) conditions. Food and water were available ad libitum. All the experiments that are shown were performed in females. Mice lacking β-endorphin carry a mutant POMC allele ($POMC^{*4}$) where the codon for the amino-terminal tyrosine of β-endorphin was changed to a premature translational stop codon (Rubinstein et al., 1993). Mice were genotyped by PCR of DNA obtained from the tail as described. Briefly, a 1.4-kb product corresponding to the WT POMC allele was amplified with 5′ primer GAAGTACGTCATGGGTCACT and 3′ primer GACATGTTCATCTCTATACATAC set of oligonucleotides. A second set of oligonucleotides, 5′ primer GAGGATTGGGAAGACAATAGCA and 3′ primer GACATGTTCATCTCTATACATAC was used to amplify a 1.2-kb band corresponding to mutant $POMC^{*4}$ allele.

### 2.2. Cell cultures

Spleens were removed aseptically from naive mice and dispensed through a metal mesh into phosphate-buffered saline solution (PBS) in order to obtain single-cell suspensions (Böyun, 1968; Raiden et al., 1995). Splenocytes were resuspended at a density of $2.5 \times 10^6$ cells/ml in a RPMI 1640 culture medium supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA), 2 mM glutamine, $10^{-5}$ M mercaptoethanol and penicillin (100 U/ml), and plated (200 μl/well) in 96-well flat bottom microtitter plates. Cultures were incubated at 37 °C in a humid atmosphere of 5% $CO_2$ for 72 h. For macrophage enrichment, total splenocytes were cultured for 120 min at 37 °C, 5% $CO_2$. Next, the nonadherent mononuclear cells were removed by washing twice with fresh medium. This procedure was repeated after an additional 120 min. This procedure yields a cell preparation that is highly enriched in macrophages (>95%), assessed by immunofluorescence (Arzt et al., 1992) using specific CD2, CD4, CD8, CD14 and CD19 monoclonal antibodies (Pharmingen, San Diego, CA, USA) which define antigens on murine T cells/NK cells, T-helper lymphocytes, T-cytotoxic/suppressor lymphocytes, monocytes/macrophages and B cells. Cell viability was always >95% as determined by trypan blue exclusion.

### 2.3. DNA synthesis assay

Splenocytes were plated at $2.5 \times 10^6$ cell/ml (200 μl/well) in 96-well flat bottom microtitter plates and were stimulated at the beginning of the culture with the following mitogenic stimuli: a T-cell mitogen, concanavalin A (Con A; 2.5 μg/ml); a T-independent B-cell mitogen, lipopolysaccharide from *Escherichia coli* (LPS; 10 μg/ml); and a T-dependent T-cell and B-cell mitogen, phytolacca americana lectin (pokeweed mitogen, PWM; 10 μg/ml) (all from Sigma, St. Louis, MO, USA). Cell proliferation rate assessed by DNA synthesis was measured by [$^3$H]-Thymidine incorporation (Raiden et al., 1995). Fresh splenocytes was cultured over 72 h in the presence or absence of mitogens and 1 μCi of [$^3$H]-Thymidine/well (NET O27 Spec. act., 6.7 Ci/mmol; New England Nuclear, Boston, USA) was added during the last 16 h of incubation. Radioactivity incorporated by the cells was recovered after extensive washing with a cell harvester (Skatron systems, Norway) and measured in a scintillation solution in a Beckman (Fullerton, CA, USA) scintillation counter.

### 2.4. Cytokine determinations

TNF-α levels in supernatants were assessed in a bioassay based on cytotoxicity exerted by TNF-α on the L-929 cell line as previously described (Arzt et al., 1991). Briefly, monolayers of L-929 mouse fibroblasts were incubated in 96-well flat bottom microtitter dishes for 24 h at 37 °C, 5% $CO_2$, with D-MEM (Gibco Laboratories), supplemented with 2% FCS. After this period, the medium was removed and replaced by fresh medium with 2% FCS and actinomycin-D (Sigma) at 0.5 μg/ml. Serial two-fold dilutions of test samples and a TNF-α standard (R&D, Minneapolis, MN, USA) were then plated in duplicate in a volume of 100 μl. After 24 h of incubation at 37 °C, 5% $CO_2$, the samples were removed; the cells were stained with 0.5% gentian violet in 70% methanol and the staining intensity was assessed by spectrophotometry at 570 nm. For TNF-α characterization mixtures of serial dilutions of positive TNF-α test samples and a constant dilution of anti-mouse TNF-α antiserum (R&D) were incubated for 1 h at 37 °C. After the incubation period, the residual TNF-α was titrated in the same way. TNF-α titration without antiserum was done in parallel.

IL-6 levels in plasma and cell-culture supernatants were measured by a specific IL-6 mouse ELISA (R&D). The inter- and intra-assay coefficients of variance were 4.4% and 7.1%, respectively, with a detection limit of 3.1 pg/ml.

For IL-2 mRNA measurement, total RNA was extracted from splenocytes and reverse transcription was carried out as previously described (Paez Pereda et al., 2000). Radioactive polymerase chain reaction was performed under restrictive conditions using only 25 amplification cycles (1 min, 94 °C; 2 min, 63 °C; 1 min 72 °C) in order to obtain band intensities proportional to the amount of RNA present in the samples (Paez Pereda et al., 2000). Actin was amplified from the same samples under the same conditions as an internal control. The primer sequences were: IL-2: 5′ TACAGGTGCTCCTGAGAGGG, 3′ GGCTCATCATCGAATTGGCAC; actin: 5′ TGACGGGGTCACCCACACTGTGCCCATCTA, 3′ CTAGAAGCATTTGCGGTGGACGATGGAGGG. RT-PCR products were electrophoresed in 6% acrylamide native gels. Radioactivity in the bands was measured in a scintillation counter.

### 2.5. In vivo studies

In vivo experiments were performed as previously described (Karanth et al., 1997; Reul et al., 1994). For both genotypes, two groups were studied: (a) sham group: control mice were injected intraperitoneally (i.p.) with saline solution and were sacrificed at the same time points as the treated group. (b) Treated group: mice were injected i.p. with LPS (1 mg/kg) (Sigma) and sacrificed 3 h after injection. In all experiments, animals were sacrificed at 9:00–10:00 AM. To control for nonspecific stress, experiments were performed under rigorous undisturbed housing conditions. Mice were anesthetized during 20 s in a glass box containing a saturated $CO_2$ atmosphere after that the animals were decapitated and trunk blood was collected in ice-chilled EDTA-coated tubes

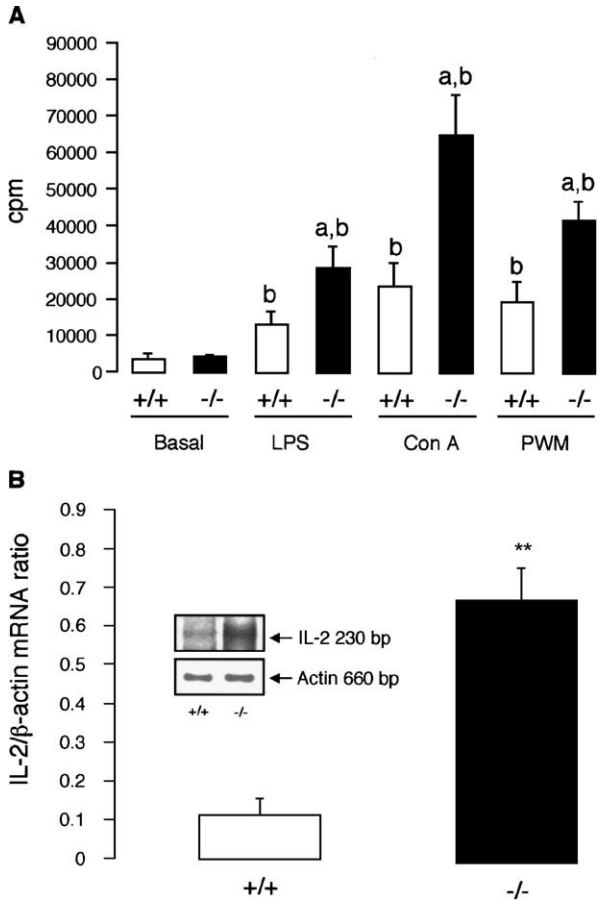


Fig. 1. Splenocyte proliferative response and IL-2 mRNA levels. (A) Splenocytes from β-endorphin deficient (−/−) and WT (+/+) mice were cultured at a density of $2.5 \times 10^6$ cells/ml and stimulated with concanavalin A (Con A; 2.5 μg/ml), lipopolysaccharide from *E. coli* (LPS; 10 μg/ml) and phytolacca americana lectin (pokeweed mitogen, PWM; 10 μg/ml) for 72 h. [$^3$H]-thymidine/well (1 μCi) was added during the last 16 h of incubation. a = $p < 0.05$ compared to the corresponding WT, b = $p < 0.05$ compared to the corresponding basal, ANOVA with Scheffé's test. Con A stimulation was also tested at 48 h and showed similar results. Mean ± SE, each condition for each animal was analyzed by quadruplicate, one representative experiment is shown. Similar results were obtained in five independent experiments. Results in females are shown; similar results were obtained with males. (B) IL-2 mRNA levels were assessed by radioactive RT-PCR. Splenocytes from β-endorphin deficient (−/−) and WT (+/+) mice were cultured at a density of $2.5 \times 10^6$ cells/ml and stimulated with Con A (2.5 μg/ml) for 12 h. RNA was extracted and RT-PCR reactions were carried out, electrophoresed and radioactivity in the bands was measured in a scintillation counter. ** $p < 0.01$ compared to WT mice, ANOVA with Scheffé's test. Mean ± SE, $n = 5$ animals each group. Inset: IL-2 and β-actin RT-PCR products of one representative (−/−) or (+/+) mice are shown.

containing 140 μg aprotinin (Trasylol, Bayer, Koln, Germany).

### 2.6. Hormone measurements

Plasma samples for ACTH and corticosterone were stored at −80 and −20 °C, respectively, prior to their measurement by RIA (ICN Biomedicals, Costa Mesa, CA, USA). The inter- and intra-assay coefficients of variance for ACTH were 7% and 5%, respectively, with a detection limit of approximately 2 pg/ml. The inter- and intra-assay coefficients of variance for corticosterone were 7% and 4%, respectively, with a detection limit of 1.5 ng/ml.

### 2.7. Statistical analysis

Statistics were performed using two-way ANOVA, with post-hoc Scheffe's test. $P$ values $< 0.05$ were con-

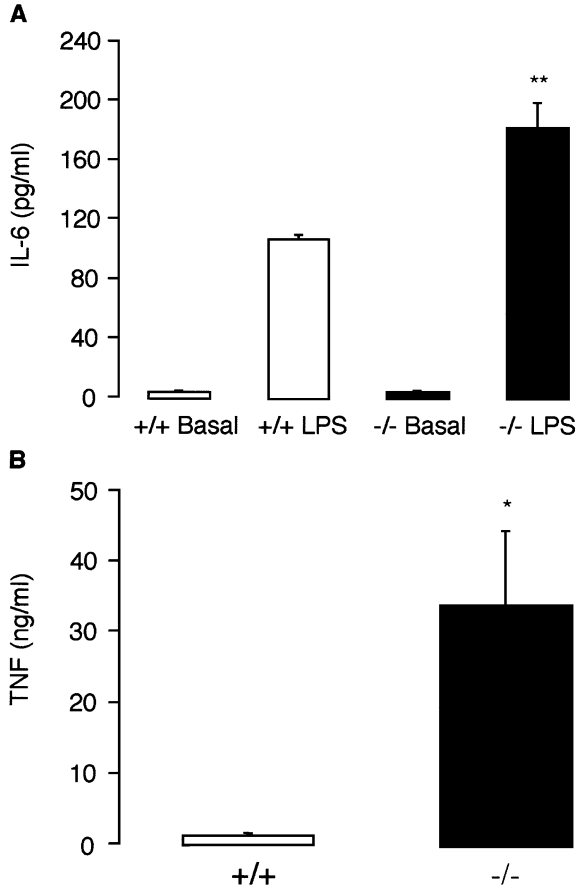


Fig. 2. IL-6 and TNF-α production by splenocyte macrophage cultures. (A) Macrophage splenocytes from β-endorphin deficient (−/−) and WT (+/+) mice were cultured and stimulated with lipopolysaccharide (LPS; 5 μg/ml) from *E. coli* for 4 h. IL-6 was measured in the supernatants by ELISA. ** $p < 0.01$ compared to all other groups, ANOVA with Scheffé's test. Mean ± SE, $n = 4$ animals each group, each condition was analyzed by quadruplicate in each animal. (B) Macrophage splenocytes from mice lacking β-endorphin (−/−) and WT (+/+) mice were stimulated with lipopolysaccharide from *E. coli* (LPS; 5 μg/ml) for 4 h. TNF-α was measured in the supernatants by L-929 cells biological assay as described in Materials and methods. * $p < 0.05$ compared to WT (+/+), ANOVA with Scheffé's test. Mean ± SE, $n = 5$ animals each group, each condition was analyzed by quadruplicate in each animal. Supernatants of untreated macrophage cultures from both β-endorphin deficient and WT mice showed no detectable TNF-α values.

sidered significant. Results are expressed as the mean ± SE.

## 3. Results

### 3.1. Splenocyte proliferative response and IL-2 mRNA levels in mice lacking β-endorphin

Splenocytes obtained from WT mice and mice lacking β-endorphin were analyzed and showed no differences in cell number/distribution and basal proliferative rates between genotypes. However, when stimulated with different mitogens, the proliferative response of splenocytes from β-endorphin-deficient mice was significantly higher compared to WT mice in response to each of the mitogens (Fig. 1A). The largest fold-increase compared to WT splenocytes was evident for Con A, which activates T cells. Accordingly, IL-2 mRNA levels were higher in Con A-treated splenocytes from β-endorphin deficient compared to WT mice (Fig. 1B).

### 3.2. Splenocyte and plasma inflammatory cytokine production in mice lacking β-endorphin

Inflammatory paradigms (i.e. the macrophage production of pro-inflammatory cytokines) were next evaluated in splenic cultures. IL-6 production in response to LPS was significantly increased in macrophage-enriched cultures from mice lacking β-endorphin compared to WT mice (Fig. 2A). Similarly, TNF-α production was also markedly increased in cultures from homozygous deficient mutant mice (Fig. 2B). After LPS administration in vivo, IL-6 plasma levels were increased in both genotypes, but were significantly higher in mice lacking β-endorphin compared to WT controls (Fig. 3).

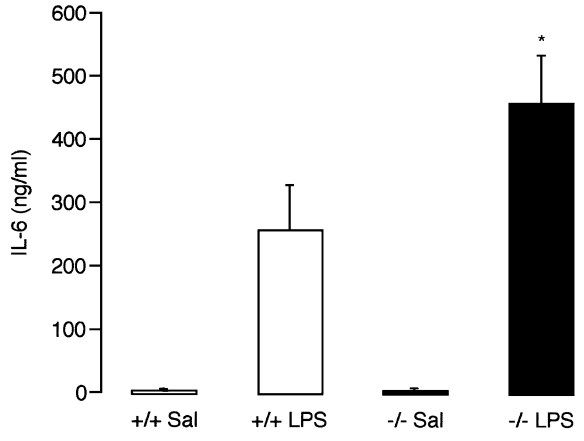


Fig. 3. IL-6 plasma levels in response to LPS. IL-6 plasma levels were measured by ELISA in β-endorphin deficient ( − / − ) and WT (+/+) mice. Plasma samples were obtained at 10:00 AM, 3 h after a single LPS (1 mg/kg) or saline (Sal) injection. *$p < 0.05$ compared to WT (+/+) LPS, ANOVA with Scheffé's test. Mean ± SE, $n = 4$ animals each group.

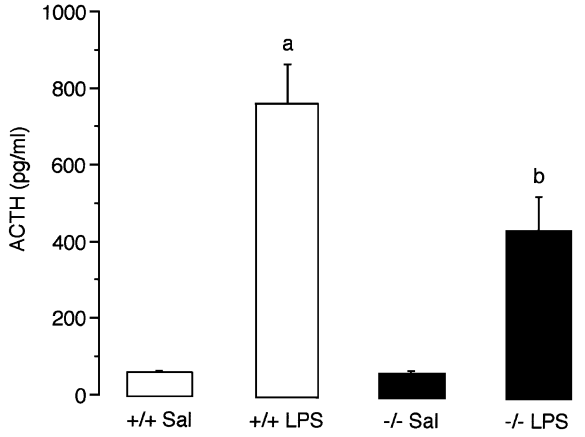


Fig. 4. Plasma ACTH levels in response to LPS. Plasma ACTH levels were measured by RIA in mice lacking β-endorphin ( − / − ) and WT (+/+) mice. Plasma samples were obtained at 9:00–10:00 AM, 3 h after a single LPS (1 mg/kg) or saline (Sal) injection. a = $p < 0.05$ compared to all other groups, b = $p < 0.01$ compared to the ( − / − ) Sal, ANOVA with Scheffé. Mean ± SE, $n = 7$ animals each group.

### 3.3. ACTH and corticosterone levels in response to LPS in mice lacking β-endorphin

We next evaluated the HPA axis response to an immune challenge in WT and β-endorphin-deficient mice. Basal plasma ACTH and corticosterone levels showed no differences between genotypes as previously reported (Rubinstein et al., 1996). In order to control the possible stress influence of the i.p. injection itself, undisturbed and uninjected mice were evaluated too. No differences were found in basal ACTH or corticosterone levels between uninjected and saline injected WT or β-endorphin-deficient groups of mice (not shown). However, the stimulated responses of both

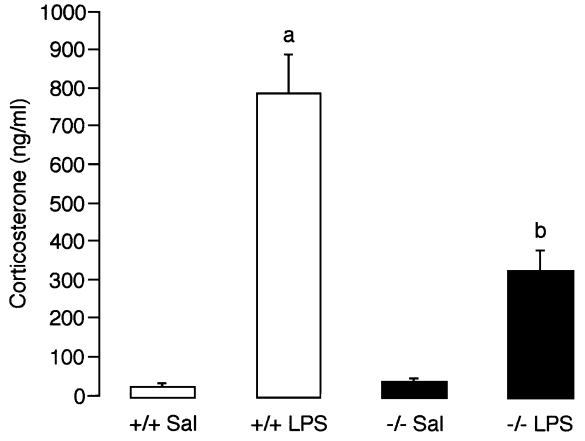


Fig. 5. Plasma corticosterone levels in response to LPS. Plasma corticosterone levels were measured by RIA in mice lacking β-endorphin ( − / − ) and WT (+/+) mice. Plasma samples were obtained at 9:00–10:00 AM, 3 h after a single LPS (1 mg/kg) or saline (Sal) injection. a = $p < 0.05$ compared to all other groups, b = $p < 0.01$ compared to the ( − / − ) Sal, ANOVA with Scheffé. Mean ± SE, $n = 7$ animals each group.

plasma ACTH and corticosterone levels to systemic LPS were significantly attenuated in mice lacking β-endorphin compared to WT mice (Figs. 4 and 5).

## 4. Discussion

To investigate the role of β-endorphin in neuroendocrine immune regulatory pathways, we have studied mice lacking β-endorphin (Rubinstein et al., 1996). We found that these mutant mice possessed an enhanced immune response measured in terms of splenocyte proliferation to a range of mitogens, splenic macrophage inflammatory cytokine production and plasma IL-6 secretion. In addition, they had a blunted HPA axis response to stimulation by LPS. These results are consistent with an inhibitory role of endogenous β-endorphin on the immune system and its instrumental participation in the neuroendocrine–immune circuits.

The proliferative response of splenocytes from β-endorphin-deficient mice was significantly higher compared to WT mice, following treatment by a T-cell mitogen (Con A), a T-independent B-cell mitogen (LPS) and a T-dependent T-cell and B-cell mitogen (PWM). These results point to endogenous β-endorphin as an important contributor to the inhibition of immune cell proliferation. As already discussed, both stimulatory (Gilman et al., 1982; Heijnen et al., 1987; Kusnecov et al., 1987; van den Bergh et al., 1991b) and inhibitory (Manfredi et al., 1993; McCain et al., 1982; Panerai et al., 1995; Puppo et al., 1985) effects of β-endorphin on lymphocyte proliferation in response to mitogens have been described. The discrepancies have been speculated to be due to donor status, diurnal variations, and time and doses of administration, although no clear conclusion could be drawn. The present work aimed to resolve these contradictory results using cultured cells that do not have the capability to synthesize, and therefore which lack the influence of autocrine or paracrine, endogenous β-endorphin. Due to the fact that (a) relative number of cells is not different, (b) basal proliferative rate is similar between genotypes and (c) the enhanced proliferation is observed with the different mitogens to which cells respond with different intensity and kinetic, the most likely explanation for the difference in proliferation rates would be a difference in the activation capability of the cells. In fact, IL-2 mRNA, the most important cytokine involved in T-cell activation is elevated in β-endorphin-deficient mice. The normal basal proliferative rate, but higher stimulation of cells from the mutant mice suggests that β-endorphin exerts an inhibitory feedback control over lymphocyte function involving helper cells.

A study using μ-opioid receptor knockout mice reported that the ex vivo morphine-mediated reduction in mitogen-induced splenocyte proliferation rate was unaffected in these mice compared to WT mice (Roy et al., 1998). This result is consistent with a naloxone-insensitive mechanism already described for opioid action on splenocyte proliferation and suggests a putative μ-independent inhibitory action of morphine. Moreover, in the absence of morphine treatment, these mice exhibited a reduction in the splenic and thymic PHA/IL-1-induced proliferation compared to WT mice, indicating that physiologically, μ-receptors may facilitate the proliferative effects induced by mitogens and that endogenous opioids act by a different mechanism compared to exogenously added morphine. Interestingly, the β-endorphin-deficient mice showed normal basal splenocyte proliferation, but a significant two- to three-fold increase in the mitogen-induced proliferation rate. Thus, the inhibitory physiological action of β-endorphin may possibly be mediated by a μ-independent mechanism.

In contrast, the LPS-induced secretion of TNF-α from peritoneal macrophages of μ-receptor knockout mice was enhanced with respect to WT animals (Roy et al., 1998). This result is similar to the enhanced LPS-induced pro-inflammatory cytokine release pattern from splenic macrophages of mice lacking β-endorphin compared to WT mice. In this context, our work helps to clarify the contradictory pro-inflammatory cytokine secretion studies that have been reported (Apte et al., 1990; Gomez-Flores et al., 2001; Hosoi et al., 1999; Peng et al., 2000).

An inhibitory role of β-endorphin on immune functions is further supported by evidence from several clinical studies and animal models. Examples of diseases in humans where β-endorphin levels are diminished are rheumatoid arthritis (Wiedermann et al., 1992), type 2 diabetes mellitus (Solerte et al., 1988), Crohn's disease (Wiedermann et al., 1994) and multiple sclerosis (Giron et al., 2000). The higher inflammatory cytokine responses in mice lacking β-endorphin suggest that the level of this peptide may be important for the onset of the inflammatory–autoimmune process, and not a consequence of its development. This reduction in β-endorphin is also evidenced in animal models of autoimmune disorders, prior to the appearance of the disease (Panerai and Sacerdote, 1997). In autoimmune onset, the enhanced cytokine production may, via stimulation of the production of β-endorphin (Cabot et al., 1997; Kavelaars et al., 1989), exert a negative feedback on the antibody secretion (Johnson et al., 1982; Morgan et al., 1990; Taub et al., 1991) and potentially on the production of autoantibodies (Panerai and Sacerdote, 1997).

Interestingly, the blunted ACTH and corticosterone responses of β-endorphin-deficient mice to LPS represent a unique differential response to stress because basal and stimulated corticosterone levels in response to restraint- and ether-induced stress were normal in the mutant mice (Rubinstein et al., 1996). This dampened response to LPS in β-endorphin-deficient mice may be a consequence of either an absence of the stimulatory basal tone or the absence of an endorphinergic pathway that participates directly in the LPS activation of the HPA system. As basal hormone levels are equal in both genotypes, the most plausible explanation may involve an β-endorphin-mediated role in LPS action on HPA axis. Thus, the low adrenal hormone levels may be a consequence of a disruption of neuroendocrine pathways by

which β-endorphin normally contributes to stimulate the CRH-driven response to LPS (Hellbach et al., 1998; Jessop, 1999; Whitnall, 1993). The diminished ACTH response to LPS could also probably contribute to the enhanced proliferation and cytokine release observed in response to LPS in β-endorphin-deficient mice.

A concomitant analysis of T-cell proliferation, inflammatory cytokine secretion and challenge-induced HPA axis activation emphasizes the concept that β-endorphin influences immune and endocrine systems in the same anti-inflammatory and immunosuppressive way. Our data also indicate that the β-endorphin regulation of neuro-immune interactions may operate via complex mechanisms, involving multiple controls acting at different levels of integration including peripheral suppression of lymphocyte proliferation and cytokine production and the central augmentation of the HPA axis and glucocorticoid secretion in response to immune challenge.

In conclusion, this work provides new evidence, derived from a study of mice deficient in β-endorphin, to support the concept that β-endorphin exerts an important and complex physiological inhibitory role on the immune system.

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